DOCTORAL DISSERTATION

The dog as a model-animal in comparative sleep spindle research

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EÖTVÖS LORÁND UNIVERSITY
2020

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There is no knowledge that is not power.

-Ralph Waldo Emerson

Acknowledgements

I would first like to thank my supervisors, of recent and past:

Dr. Enikő Kubinyi, who supervised this thesis and the efforts that are described hereafter. She trusted my ability when my curriculum vitae could not speak up for me, yet, and provided the necessary means and support for this project to work. Our written exchanges while working on manuscripts together, helped me develop ideas and map my way through the relevant literature. She took the time and patience to review and promote all ideas that came to my mind, while making sure the most urgent goals do not leave our sight. **Dr. Gilles van Luijtelaar**, who supervised my bachelor and master theses. Who took the time to discuss neuroscience on almost philosophical levels of depth and continued to support me, morally and academically, long after his official role as my teacher had ended. His dry and confrontational, yet cordial humor were always uplifting the spirit of our interactions. **Dr. Hein van Schie** who supervised my first literature study in psychology and continued to explore ideas with me long after I left the Radboud University of Nijmegen. One of our literature studies became my first publication, which helped me regain confidence in a difficult time. **Dr. Torfi Sigurdsson**, who after a time that was hard for both of us, did not take it personal that we were a bad match.

From the senior university teachers/professors with whom I interacted, but were not my direct/official supervisors I would like to thank:

Dr. Arno Koning who first taught me academic writing, the late **Dr. Jacques Janssen**, **Dr. Pieter Medendorp** and his then PhD students, now **Dr. Frank Léoné** and **Dr. Ivar Clemens** who taught me to use Matlab, our faculty's Statistics professor **Dr. Jules Ellis**, **Dr. Tineke van Rijn** for knowing how to ignite interest in brain anatomy and physiology, all of above associated with the Radboud University of Nijmegen. **Dr. Jochen Roeper** who kept up the intellectual spirit in the otherwise worldly Frankfurt. In the ELTE department of ethology I also had the honor to meet and learn from **Dr. Ádám Miklósi**, **Dr. Attila Andics** (whose efforts in organizing the department retreats gave me a chance to socialize more directly with many colleagues), **Dr. Péter Pongrácz** (whom I thank a visit to a life animal market and my first experiences as a peer-reviewer), **Dr. Ákos Pogány** and **Dr. Tamás Faragó** (these two I want to also thank specifically for engaging the department in short-lived, but fun musical projects). I am also grateful for meeting **Dr. Claudia Fugazza** and **Dr. Márta Gácsi** and having the opportunity to ask some direct questions about their interesting lines of work. A very special thanks also to **Dr. Dorottya Júlia Ujfalussy**, for helping out with some highly relevant bureaucratic pressures right in the beginning of my stay (but in that regard also I want to mention and thank **Boróka Bereczky**, **Ágnes Modrovics** and **Borbála Győri**).

I would next like to thank a few personal friends from within academia, who at times unofficially acted as "secondary" or "supporting" supervisors, as well as those who worked with me towards shared goals:

Specifically with regards to EEG/physiology these were **Dr. Anna Vargáné Kis, Dr. Róbert Bódizs, Dr. Annika Lüttjohann,** and **Dr. Péter Przemysław Ujma**. A special thank you within this category goes to **Dr. Kauê Machado Costa**, who not only was the supervisor I

needed during my time in Frankfurt, but a friend and colleague for life with whom I hope to share more cold Guinness and exciting cooperations in the future. Working hard with me on data-collection for this thesis were fellow students **Dóra Szabó**, **Vivien Reicher**, **Daniel Rodrigo Tejeda Fernández de Lara**, **Zsófia Bognár** (some of which are now also co-authors on several of my publications) and for helping organize the obtained data a very special thanks goes out to **Dr. Borbála Turcsán**. In ethology I have also learned a lot from working with **Dr. Lisa Wallis** and **Dr. Patrizia Piotti**. I want to also thank **Dr. Raúl Hernández** and **Dr. Laura Verónica Cuaya** in advance for their willingness to help translate to action one of my oldest research ideas, whether or not this succeeds in the long run. Back when studying psychology it was my former study mate, now PhD as well, **Dr. Willem Sleegers** who occasionally taught me important aspects of good work ethics and psychological experimentation and was one of the first to remind me of my research dreams as well as to love the questions more than the answers.

While still in the domain of the professional, I would like to thank colleagues who contributed to a pleasant and interesting work atmosphere:

Dr. Kálmán Czeibert, **Dr. Sára Sándor**, **Dr. Boglárka Erdélyi-Belle**, **Boglárka Morvai**, **Dr. Judit Abdai**, **Dr. Nóra Bunford**, **Rita Báji**, **Petrouchka Hulsbosch**, **Barbara Csibra**, **Nikolett Jégh-Czinege**, **Tímea Kovács**, **Andrea Piseddu**, **Enrica Aguzzoli**, **Louis Le Nézet**, **Laura Gillet**, **Iris Smit**, **Etkin Şafak, Soufiane Bel Rhali**, **Ilona Tarnowska, Dr. Marianna Boros, Dr. Lilla Magyari, Brian Rummell, Jan Klee, Alois Kreuzer, Pascal Vogel, Beatrice Fischer, Jasmine Sonntag, Lora Kovacheva, Navid Farassat, Johannes Hahn, Dr. Sebastian Betz, Thomas Wulf**, **Günther Amrhein**, **Regina Giegerich**, **Jarrod Dowdall**, **Dr. Valerio D'Amore**, **Dr. Mehrnoush Zobeiri**, **Dr. Ingar Zielinski**, **Dr. Lili Huang**, **Martin Perescis**, **Dr. Magdalena Smyk**, **Dr. Wioleta Walentowska**, **Dr. Emanuel van den Broeke**, and **Dr. Jeff** – the philosopher – **Martin**.

Coming to the realm of the personal. I want to thank my mother **Tinka Siepmann-Iotova**, my grandmother **Vichka Iotova** and my late grandfather **Ivan Iotov**, who together formed the first family I knew. Not far from them, my father **Borislav Iochev**, who worked hard to maintain a functioning relationship with me, his sister (my aunt) **Joanna Savova**, my uncle **Milcho Savov**, and their daughters (my cousins) **Vesela** and **Petya** for forming a close and mutually supportive (with regard to my upbringing) network with my mother's family, becoming an important presence during my childhood. Next, my wife **Leonora Iotcheva** and our daughter **Lyka**, with whom we have begun a new chapter in the family's history and who magically make me feel at home anywhere they are. Among my blood relatives I also owe special gratitude to **Yasen Georgiev**, **Elena Michailova**, **Fani Georgieva**, who are not only my cousins, but also my childhood friends, as well as my paternal grandparents **Vesela** and **Trifon**. Last, but not least in this category I want to thank/acknowledge the youngest of the clan, my brother **Hennes Siepmann**, my late brother **Philipp Siepmann**, and my sisters **Eline Langmyr-Iochev** and **Tyra Langmyr-Iochev**.

Next comes the non-related family, my bloods-brother **Max Krys**, the mother of my godchild **Dr. Kim Morawa/Doht** and my godchild **Tomke Doht**, the mother of my sisters **Guri Langmyr-Iochev**, my step grand-parents **Helmut** and **Gertrud Siepmann**, their sons/my step-uncles **Jörg** and **Frank Siepmann**, my step-cousins **Tobias**, **Alina** and **Mascha**. As well as

friends which became friends of the entire family: **Fabian** and **Sandra Kaul**, **Andreas Sieber** and **Cem Günay**.

I would then like to thank all my close friends and in general inspiring people who crossed paths or are still walking with me. The closest among them who are neither related nor colleagues are **Arne "the raven" Scheinpflug**, **Simone Riemann**, **René Verheesen**, **Ines Schürmann**, **Timo Bretten**, **Alexandra** and **Lyubo Bibishkovi**, **Lyuben Laskin**, **Meryl Varadinov, Filip Świderski** and **Martyna Krajewska**, **Zuzanna Szczerbanowska**, **Ewa "the fox" Lis**, **Justyna Janik**, **Bence Faragó**, **Monika Wintermanns**, **Zalán Anga**, **Mihály Fodor**, **Béla Mucs** (and his lovely family), **Izabel Kempf** and her sister **Anni Dembinski**. But also many others who made this journey special, including, but not limited to: **Carlotta Giustozzi**, **"Von Chrisbert"** and the other **bicycle cossacks**, **Simon Holthausen**, **Lisa Anders**, **"Steve Rick"**, **Philipp Sipano**, **Ünal Deniz** and **"Mark"**, **"Derrick Rake"**, **Rianne Luiten**, **Sam Bruijs**, **Daniel Buize**, **Jan Liket**, **Benedict Stein**, **Arvid Hüffmann**, **Jona Heidsick**, **Daniel Lämmerhirt**, **Mara Cofalla**, **Toni Matzulla**, **Janine Wilting**, **Ann-Kathrin Dresemann**, **Falko Hoffmann**, **Melissa Schüller**, **Henning "honing" Mert**, **Bas** and **Samira Bevers**, **Matthias Wetzel**, **"Mido"**, **"NN"**, **Louise "Lemon"**, **Anna-Lena Hoh**, **Bijou** (not the dog, although also a nice person), **Michael Stadler**, **Dr. Thomas Wolfers**, **Jana "Jaana"**, **Ahmet Kaya**, **Yusuf Celik**, **Dalia Samur**, the late **Romina Aciman**, **Theo Kosters**, **Manuel "Malenki"**, **Niklas Ganesha** and **Elena Sternschnubbi**, **Yaşatan Günay**, **Orlin Todorov**, **Dr. Wendy van Ginkel**, **Dr. Bronagh McCoy**, **Laura Verheesen**, **Yvonne Verheesen**, **Dr. Ashley Lewis**, **Agata Koim**, **Marusha Kos**, **Aga Kavak**, **Dr. Ania Pitas**, **Robin Lambertz**, **Jenni Berndt**, **Daniel Knippschild**, **Vera Knippschild**, **Sara Junk**, **Daniel "Punk"**, **Daniel "Jürgen"/"Jorgi" Jahnke**, **Thomas "Nocks" Wagner**, **Jannis Flieger** and **Ellen Franken**, **Tobias "Flay" Flüs**, **Felix Seidenfaden**, **Katharina Klupsch**, **"Rockgig" Dan**, **Tim "Meister" Demetry**, **Marius Sommerfeld**, **Hendrik Thus**, **Hendrik Kleefisch**, **Simon von Eyser**, **Rainer Elschen**, **Human Riahi**, **Sara Świerkosz**, **Dr. Katalin Szlukovényi**, **Anna Wilhelm**, **Anna Szokolay**, **Norbi Dudás**, **Adri Szabó**, **Ildi Hajdu**, **Jakab Daróczi**, **Rebeka "Rebus" Kasuba**, **Ágoston Lehoczki**, **Zoltán Molnár**, **Ferenc Korpai**, **István Korcsmáros**, **István András Badacsonyi**, **István Czeti**, **Virág Koller**, **Rita Tamics**, **Ferenc Pusztai**, **Miklós Gula**, **István Várró**, **Norbert Luksa** and **Anna Hegyi**, **Dániel Keliger**, **Anna Kerti**, **Kórnelia Bán**, **László Bajzáth**, **Attila Boros**, **Lázár Pap**, **Franciska van Waarden**, **Borbála Zsilák** and **Gábor Fazekas**, **Tamás Bálint**, **Mercédesz Bauman**, **Fanni Alexandra Heim**, **Viola Németh**, **Rita Lápossy**, **Nóra Solymosi**, **Viola Csérveg**, **Sára Ungvári**, **Ábel Kocsis**, **Csanád Szabó**, **"Goth" Alex**, **Gábor "Németh" Varga** and **Eszter Mónok**, **Timea Török**, **Márk Török**, **Sonja Zając**, **Christina "Vuki" Vukova**, **Tsvyatko Hristov**, **Svetlozar Kalchev**, **Miroslav Damyanov**, **Damyana Stoyanova**, **Iván Kubinyi**, **Attila Molnár**, **Yasemin Orlu**, **Agata "Mi"**, **Mehmet**, **Harry Pierce**, **Mario Butkovic**, **John Morovich**, **Jelena** and **Svetlana Juric**, **"Gino" Srdjan Yevdjevich**, **Dimitar Egumenovsky**, **Diana Rosentul**, **Mirco** and **Yvonne Meyer**, **Nils Achterfeld**, **Daniel Rettinghausen**, **Fabiola Gerpott**, **Birk Hahne**. Finally, two people who pushed me in the right direction (for the wrong reasons), yet I do not associate with anymore: **Lisa Elschen** and **Dirk Siepmann**.

Finally I would also like to thank the funding institutions which enabled this project: the European Research Council (ERC) under the European Unions Horizon 2020 research and innovation programme (Grant Agreement No. 680040), **Nestlé Purina**, **the BIAL foundation**, **the Hungarian Scientific Research Fund**, and **the Hungarian Brain Research Program (**2017-1.2.1-NKP-2017-00002).

Abstract

In dogs a focus on epilepsy and the macrostructure of sleep has dominated the EEG literature for decades. Both are holistic changes in the entire brain and cannot always be traced back to specific loci and mechanisms. Sleep spindles appear in the EEG of humans, rats and cats as brief bursts (0.5-5 seconds) of relatively fast (> 9 Hz, maximally 16 Hz) oscillations against the background of slower and larger waves characterizing non-REM sleep (1-4 Hz). They have a well-understood underlying mechanism and are implicated in various brain states and capacities, including memory, intelligence and sleep stability, while their features also change with age and the menstrual cycle. This makes sleep spindles ideal candidates for widening the scope of canine EEG.

In this thesis I have sought to gather arguments, across two data sets, for the validation of the dog as a model animal in sleep spindle research and for the validation of a spindle-detection method I have introduced for this species. To avoid a circular reasoning error in which the answer to each question is the sole support for the other, I investigated a limited set of hypotheses on the frequency of canine spindles, while also keeping other search criteria similar to what is established for humans.

Comparable studies in humans and rats suggest that spindles should increase in density (occurrence/minute) after exposure to novel information and that density correlates positively with recall across species that express them. Both predictions were satisfied specifically for detections in the 9-16 Hz range in a sample of 15 dogs, suggesting that the characteristic frequency of human and dog spindles is similar. Moreover, we observed that female dogs expressed more sleep spindles and performed better on the novel task than males.

A follow-up study on 155 dogs suggested furthermore that spindles in the dog can be subdivided into a slow (≤ 13 Hz), anterior and a fast (≥13 Hz), posterior subtype; that the amplitude, density and frequency of the fast subtype is higher in females and that the amplitude and density of the slow type decreases with age. These findings align with what is known from humans and rats, but an increase in fast spindle density with age was unique to dogs and stimulates exciting questions for future research. The here presented sleep spindle detection instrument was validated by replications across the two studies and by cross-species replications.

Samenvatting (Summary in Dutch)

De EEG literatuur in honden heeft in de laatste decennia vooral aandacht besteedt aan epilepsie en de macro structuur van slaap. Beide zijn holistische brein veranderingen en de onderliggende mechanismen en locaties zijn niet altijd duidelijk. Slaap spullen verschijnen in het EEG van mensen, ratten en katten als korten (0.5-5 sekonden) en relatief snelle (> 9 Hz, maximaal 16 Hz) oscillaties tegen de achtergrond van langzaamere en grootere golven (1-4 Hz) bepaalend voor non-REM slaap. De onderliggende mechanismen zijn wel bekend en zijn geimpliceerd in het vormen van herinneringen, intelligentie en de stabiliteit van slaap. Verder veranderen de eigenschappen van slaap spullen tijdens de menstruatie en met groeiende leeftijd. Daarom zijn slaap spullen ideaal om het nut van EEG in honden te verbreden.

In deze dissertatie presenteer ik argumenten, verzameld over twee studies, voor het valideren van de hond als model dier in onderzoek naar slaap spullen, als ook voor de hier gebruikte automatische detector. Om een cirkelredenering te voorkomen, waaring de antwoort op elke van deze vraagen de enkele steun is voor de ander, heb ik mij a priori beperkt tot drie hypothesen over de frequentie van slaap spullen in de hond, terwijl andere zoek-criteria aangepast zijn aan wat in mensen gebruikelijk is.

Studies in zowel mensen als ook ratten suggereren dat in soorten die slaap spullen vertonen, het aantal slaap spullen per minuut groeit bij het blootstellen aan nieuwe informatie, en ook positief is gecorreleerd met geheugen prestatie. Allebij werd in 15 honden getoond alleen wanneer slaap spullen als 9-16 Hz oscillaties werden gedefinieerd. Dit suggereerd een vergelijkbare frequentie tussen de slaap spullen van mens en hond. Verder vertonden vrouwelijke honden meer slaap spullen en betere prestatie op de nieuwe tak.

Vervolgens kon ik in 155 honden aantonen dat een verschil tussen anterieure, langzaame (≤ 13 Hz) en posterieure, snelle (≥ 13 Hz) slaap spullen bestaat; dat het aantal snelle slaap spullen, zowel als hun frequentie en amplitude groter was in vrouwelijke honden en dat het aantal en amplitude langzaame spullen met leeftijd afneemt. Deze befindingen ontspreken wat in mensen en ratten bekend was, maar een leeftijd afhankelijke groei van snelle slaap spullen was uniek voor de hond en stimuleert interessante vragen. Gerepliceerde bevindingen tussen de twee studies en tussen de hond en andere soorten valideert verder ook het hier gepresenteerde detectie instrument.

Összefoglalás (Summary in Hungarian)

Az EEG kutatás évtizedeken keresztül elsősorban az epilepsziára és az alvás makrostruktúrájára irányult. Mindkét jelenség a teljes agyműködést érinti, így nem mindig lehet visszavezetni őket egyetlen specifikus régióra, valamint mechanizmusra. Az alvási orsók rövid (0,5-5 másodperces), viszonylag gyors (9-16 Hz) oszcillációk a non-REM alvás lassú és hosszú hullámformái között, emberek, patkányok és macskák EEG görbéjében. Mechanizmusuk jól ismert, valamint az is, hogy szerepet játszanak a memória, intelligencia, alvási stabilitás kialakításában, változnak korral és a menstruációs ciklus során. Emiatt az alvási orsó ideális jelölt a kutya-EEG területének növelésére.

Disszertációmban két adatsor elemzése alapján amellett érvelek, hogy a kutya valid modell az alvási orsó kutatásában, és az általam kidolgozott módszer valóban orsókat detektál. Annak érdekében, hogy ne essek a körkörös érvelés hibájába, ami során egy következtetést a bizonyítandó állítás igaznak feltételezésével igazolok, korlátozott számú hipotézist vizsgáltam az orsók frekvenciájával kapcsolatban, és az orsók keresének elvét az emberekre kidolgozott kritériumokra alapoztam.

Embereken és patkányokon végzett kutatások szerint az orsók denzitása (előfordulás/perc) új információ hatására növekszik, és pozitívan korrelál azzal, hogy az egyed milyen hatékonyan képes felidézni az új információt. Mindkét predikció alátámasztást nyert 15 kutyából álló mintánkban, specifikusan a 9-16 Hz-es tartományban, ami arra utal, hogy a kutyában és emberben hasonló az orsók frekvenciája. Ezen kívül a szuka kutyákban több orsót figyeltünk meg, és az új feladatot is jobban teljesítették, mint a kanok.

A következő tanulmány 155 kutyán arra utalt, hogy az orsók feloszthatók lassú (≤ 13 Hz), anterior és gyors (≥13 Hz), posterior altípusra. A gyors altípus amplitúdója, denzitása és frekvenciája magasabb szukákban, míg a lassú altípus amplitúdója és denzitása a kor előrehaladtával csökken. Ez megfelel annak, amit embereknél és patkányoknál mértek, ugyanakkor a gyors orsók denzitásának korfüggő növekedése csak a kutyákra jellemző, ami izgalmas kérdéseket vet fel jövőbeli kutatások számára. A disszertációban ismertetett orsódetektálási eljárás validitását a két tanulmány és fajok közötti összehasonlítások hasonló eredményeivel igazoltuk.

Chapter 1: General Introduction

1.1 Outline

The present thesis, submitted for obtaining the doctoral degree in the discipline of ethology is specifically concerned with the *sleep spindle* – a prominent object of research in sleep physiology. Sleep physiology itself is not the first area of investigation that comes to mind in the context of ethology, a discipline that originates in the observation of naturally occurring animal (including human) behaviors, under preferably natural conditions (Tinbergen 1963). Sleep is defined on the behavioral level by a relative absence of responsiveness (Van Twyver 1969; Zepelin 1970) and an absolute absence of (goal-directed) behavior (Kryger et al. 2011). Meanwhile physiological investigations also often require rigorous experimental manipulation that removes the subject from its natural environment[1].

The argument for ethologists to attend to questions of sleep physiology is two-fold. First, brain processes occurring during sleep have consequences for awake behavior and performance: one proposed function of sleep is long-term memory consolidation (Genzel et al. 2014), the process by which short-term memories transform from hippocampus-dependent transient associations to permanent structural changes of the cortex (Tse et al. 2007). Second, the relatively recent cognitive revolution which affected both psychology (Pekala 2013) and ethology (Kamil 2007) has more generally prompted interest in neuroscience and physiology among researchers of behavior.

In order to better understand what a sleep spindle is and how and what brain activity is measured when we focus on sleep physiology, I will begin the introduction to this work with a comprehensive overview of EEG[2] as a measurement tool. I will then discuss the underlying physiology of the phenomenon called *sleep spindle*, which we can observe with EEG in the sleeping brain.

1.2 What is EEG and what does it measure?

Among the many homeostatic processes found in biological systems, the maintenance of a stable electrical potential, i.e. the difference in electrical polarity between two points of

[1]This is particularly true for a large body of invasive work in laboratory animals, of which the classical studies of Steriade and colleagues (cited later in this chapter) were specifically dedicated to sleep physiology. Arguably even less invasive methods are a step away from natural conditions, like sleeping with an electrode-cap in a lab environment.

[2] EEG is short for electroencephalogram - Old Greek for „electrical images from inside the head" a name chosen by Hans Berger (1934) who pioneered the method in human subjects.

reference (or voltage), is particularly important for intra- and inter-cellular communication. Changes in voltage across the cell membrane act as signals from one region of the cell to another, or sometimes across different cells. Among the functions of these signals is the induction or suppression of specific cellular functions, such as muscle contraction, neurotransmitter release or the expression of certain genes. The importance of voltage change for bio-mechanical processes was first observed in the late 18th century by Luigi Galvani and his students (Galvani 1791) who could contract the muscles of a dead frog's leg using relatively weak electrical currents.

The neural system in particular has evolved to tightly control the movement of electrically charged ions across the membrane of its cells, for neural cells can only encode information precisely if changes in voltage are not the result of random fluctuations. The baseline voltage of most neurons, the cells of the neural system, is around -70 mV (relative to extracellular space) and if it rises above a certain threshold, most commonly around -40 mV, the neuron will from that point on continue depolarizing across its entire cell body (i.e. the voltage will become increasingly positive relative to extracellular space), until as a final consequence of that process it releases chemicals, called neurotransmitters, which can alter the voltage of other cells and thus enable the propagation of information (see Kolb and Whishaw (2001; 2008) for a comprehensive overview; Kandel (1989) for an in depth discussion). The thus activated neuron is said to have produced an action potential and subsequently repolarizes (returns) to -70 mV due to the homeostatic control of intra-cellular voltage (see Figure 1). In its entirety the de- and repolarization take around 4-5 milliseconds and includes a refractory period (of around 1 millisecond) during which the neuron cannot initiate another action potential.

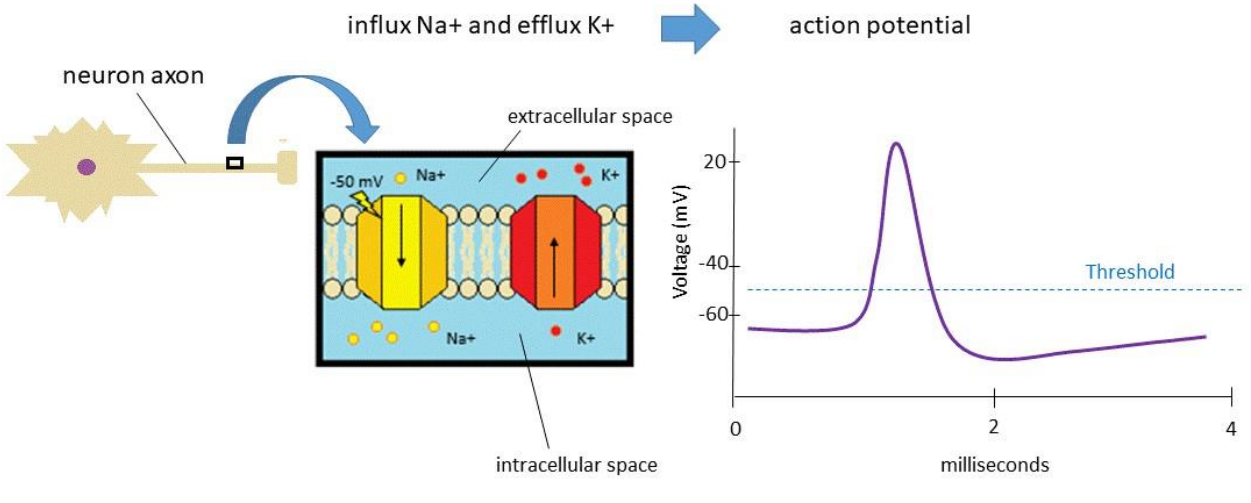


Figure 1. The voltage progression during and after an action potential, including a description of the relevant ion-flows: Na+ influx depolarizes the cell. The outflow of K+ aids a slow return to baseline.

Importantly not all signals that change the potential of a cell cause action potentials, but they can sum up over time or space (across the membrane of the targeted neuron) to reach the threshold. Some neuro-transmitters hyperpolarize the neuron by allowing more negative charge inside the cell, thereby reducing the chance for further action potentials. These perturbations of electrical homeostasis are known as EPSPs (excitatory post-synaptic potentials; synaptic refers to the synapse – the zone between two neurons where most neurotransmitters are released) or IPSPs (inhibitory post-synaptic potentials) depending on whether they increase or decrease the chance of an action potential, respectively.

EPSPs, IPSPs and action potentials are among the main forces that shape the electrical field changes around neurons that can be measured with electrophysiological instruments (Buzsáki et al. 2012). EEG is among these techniques that track changes in voltage over time caused by changes in extracellular electrical fields. While some authors differentiate LFP (local field potentials, measured directly in extracellular space with intracranial electrodes), EcoG (electrocorticogram, measured subdurally on the cortical surface), and use EEG only to denote extracranial measurements from the scalp of a subject, others might use EEG to generally describe any measurement of broadband[3] extracellular voltage across time caused by the brain's activity and specify intracranial measurements as 'intracranial' or 'invasive' EEG. Importantly, all these techniques have in common that one or several electrodes placed inside or close to the brain track voltage changes over time relative to a reference electrode of assumed stable polarity, while a third type of electrode, the ground electrode, isolates the circuit from non-biological electrical noise.

1.2.1 EEG as a window into the sleeping brain

A single EEG scalp electrode measures the electrical activity in 10 cm^2 of human cortex (Buzsáki et al. 2012). Neighboring cells within an area of this size are seldomly synchronized during wakefulness, as they participate in different networks and are involved in different tasks. Currents of opposing polarity generated by different cells at the same time within this area will cancel each other out due to the laws of wave superimposition. Due to these dynamics, EEG signals during wakefulness are both relatively flat and irregular. In contrast, large, rhythmic

[3] As opposed to single cell derived signals.

and slow (< 4 Hz) waves predominate during non-REM[4] sleep, when most cortical cells are synchronized (Figure 2). Simultaneously occurring action potentials and the EPSPs that lead up to them add up and appear as large amplitudes on the EEG recorded waves. These were first explicitly described by Blake and Gerard (1937) as a rhythm of 0.5-3 Hz (waves/second). Large-scale cortico-cortical synchronization as the underlying mechanism was demonstrated by Steriade and colleagues in the cat (1993).

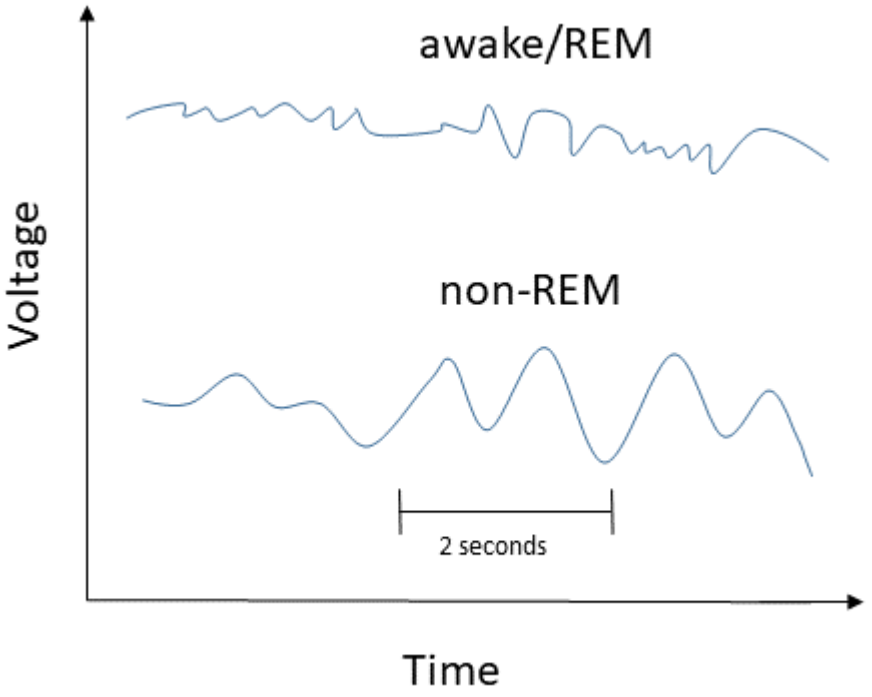


Figure 2. Sketch of the relative differences between EEG traces observed during wakefulness (REM sleep) and non-REM sleep (absolute voltage values can vary between individuals). In humans non-REM sleep can be further divided into 4 stages, not shown here. Due to a similar EEG signal REM sleep can only be distinguished from wakefulness with additional physiological measurements (muscle tone, heart-rate, and eye-movements).

EEG methods found an early application in the investigation of sleep physiology (Blake and Gerard 1937; Loomis et al. 1937; Blake et al. 1939). To the present day it is the preferred tool of sleep researchers. Its low spatial resolution does not present a problem in sleep stage identification, since the states of wakefulness/sleep present global changes across the entire brain, and the time-resolution of EEG is still better than that of the spatially more accurate MRI/fMRI (Rodden and Stemmer 2008). While EEG alone suffices to distinguish wakefulness and paradoxical (REM) sleep from deep sleep, additional monitoring of muscle tone and eye movement are necessary to distinguish wakefulness from REM. Brain activity in REM

[4] REM versus non-REM is the oldest and broadest qualitative distinction in the sleep-recorded EEG signal. The meaning of these abbreviations are explained in the next paragraph.

resembles that of the awake brain, but skeletal muscle tone is markedly reduced, while the eyes move excessively (Aserinsky and Kleitman 1953). The latter observation is responsible for the now common abbreviation REM (Rapid Eye Movement) and the modern broad distinction of REM versus non-REM sleep.

The holistic monitoring of both brain waves and body movements began with work by Blake, Gerard and Kleitman (1939) and would later become known as "polysomnography" (Deak and Epstein 2009) from ancient Greek poly (literally "many", referring to the combination of different measurements), somnium (Latin for "dream"/"sleep") and graphy (from Old Greek graphein – "image"/"depiction"). Polysomnographic methods in human patients quickly grew to include eye-movement and heart-rate monitoring (see Foulkes (1996) for a history of dream research).

1.2.2 Animal models in brain and sleep research

Much of what we know about the brain is derived from animal research. Non-human research subjects in neuroscience are predominantly from the rodent clade, specifically mice (*Mus musculus*) and rats (*Rattus norvegicus*), with mice having received slightly more attention as of recent, due to an enhanced understanding of their genome and more readily available tools for genetic manipulation (Ellenbroek and Youn 2016). Our current understanding of what might be universal principles of mammalian brain function are mostly derived from these rodent models, since until now their legal and ethical position allows for the experimental manipulations necessary to obtain this type of knowledge. Lesion studies combined with invasive recordings, sometimes on the level of a single cell, and the more recent optogenetic toolkit (Gerns 2016) have allowed researchers to investigate the causal connections between brain regions, cell clusters and individual cells of rodent brains to the execution of various behaviors and mental operations. Importantly, some of the functions thus investigated seem reasonable analogues, or at least proto-forms, of important human capacities, not shared (to our current knowledge) with even 'lower' model animals like the fruit fly *Drosophila melanogaster* (but see van Alphen and van Swinderen (2013) for a discussion of using fruit flies in the study of human psychopathology). These more 'humanlike' capacities in rats and mice include executive functions (Kolb 1984), working memory (Dere et al. 2018), categorization of learned information (Tse et al. 2007) and decision making based on cost-benefit calculations (Hillman and Bilkey 2010). Most recently, we can also observe attempts to establish rodent models of empathy (Atsak et al. 2011; Ben-Ami Bartal et al. 2014; Carrillo et al. 2019). Critical voices have warned against overemphasizing the similarities between rats and humans (Craig 2009),

but even with this caution in mind, rats and mice present an ideal intermediate solution for the integration of both scientific and ethical concerns. Research with rodents is on one hand less controversial than invasive studies in non-human primates (Abbott 2014), while as illustrated above, they display more similarities to humans in comparison to invertebrate models like fruit flies.

In the early 20th century, when invasive research with animals was less controversial, the cat emerged as a popular model animal in brain research (see discussion by Pampiglione (1971)). This model animal played a particularly important role in the study of the sleeping brain. It was in the cat that Jouvet (1979) demonstrated the first indication of dreaming in non-human animals. Deactivating the motor-inhibition characterizing REM sleep caused cats to seemingly act out upon stimuli not present in their physical environment, which was speculated to be derived from the content of their dreams. It was also in the cat that Steriade and colleagues studied and found the mechanisms behind slow oscillations (Steriade et al. 1993; Amzica and Steriade 1995) and sleep spindles[5] (Steriade et al. 1985; Bazhenov et al. 1999) characterizing the non-REM sleep phase.

Although clinical case studies (Caramazza 1988; Roser and Gazzaniga 2010) and pre-surgical brain stimulation (Penfield and Erickson 1941) have confirmed some of the animal-derived expectations formed about the causal dynamics in the human brain, the human literature is dominated by correlational observations with comparably little direct demonstration of how function is mechanistically realized.

1.2.3 The brief history of canine EEG and the importance of comparative sleep spindle research

What role did dogs (*Canis familiaris*) play in brain and (in particular) sleep research and what potential do they offer for the future? The wide use of rodent models (Ellenbroek and Youn 2016) and the historic importance of cats for sleep physiology in particular (Jouvet 1979; Steriade et al. 1985, 1993; Amzica and Steriade 1995; Bazhenov et al. 1999) seem on first glance to leave little need for the exploration of canine sleep physiology. Add to this the growing ethical concern for invasive research in dogs (Bailey and Pereira 2018) and one of the primary advantages of using animal models seems lost.

[5] It should be noted at this point, however, that earlier efforts to understand the cat thalamus played a grave role in the discoveries of Steriade et al. These are discussed in more detail in Steriade and Llinás (1988).

Even with ethical restrictions in place, however, comparative work with dogs maintains some advantages over studying humans directly: Their shorter lifespan makes it easier to conduct longitudinal studies, while the degree of inbreeding among dog breeds also eases genetic research (Kubinyi et al. 2011). While these features are shared with most research animals, dogs have been argued similar to humans in ways that overshadow the more widely used rodent model (Topál et al. 2009; Bunford et al. 2017) and which is likely a consequence of their adaptation to the anthropogenic environment (Miklósi 2014). In addition to being potentially helpful in understanding processes relevant for human medicine, dogs are themselves the target of clinical efforts and there have been attempts to transform canine EEG into a diagnostic tool for veterinarians (Croft 1962; Burcar et al. 1977; Pákozdy et al. 2012). In conclusion, dogs occupy a unique niche within the range of possible animal models.

Although research history on dog neurophysiology includes invasive work (Petersen et al. 1964; Fox 1966; Kabat and Dennis 2013) and pharmacological interventions (Wauquier et al. 1988; Pellegrino and Sica 2004; Jeserevics et al. 2007) and although laboratory dogs for terminal experiments have a considerable history of use in general, dating back as early as the 19th century (Pampiglione 1971; Field 2018), the dog brain is still by and large *terra incognita*. The study of brain function in dogs is relatively new (excluding the isolated attempts of Práwdicz-Neminski (1925) in the early 20th century) and sparse when placed in a broader context. Surprisingly little work has aimed to isolate the functional parts of dog brains from each other and determine their separate contributions to the working of the whole. So far most reported experimental manipulations interrupting the default state of dog brains are limited to anesthesia and drugs (Charles and Fuller 1956; Wauquier et al. 1988; Pellegrino and Sica 2004; Jeserevics et al. 2007; Pákozdy et al. 2012), kindling (Maars and Lopes Da Silva 1983) and intracranial (but not intracerebral) electrode-placement (Petersen et al. 1964). On the rare occasions that the manipulations involved lesions, the entire brain was deactivated or disconnected from the spine (Fox 1966; Kabat and Dennis 2013).

The state of the art knowledge of dog EEG signals can be sorted into five, partially overlapping, areas of inquiry. These are epilepsy (Maars and Lopes Da Silva 1983; Pellegrino and Sica 2004; Jeserevics et al. 2007; Pákozdy et al. 2012), sleep macro-structure (Petersen et al. 1964; Kis et al. 2017a, b; Bunford et al. 2018), states of vigilance within wakefulness (Pampiglione 1963, 1971; Wauquier et al. 1988), effects of anesthesia and drugs (Wauquier et al. 1988; Pellegrino and Sica 2004; Jeserevics et al. 2007; Pákozdy et al. 2012), and early (comparative) brain development (Pampiglione 1963, 1971; Petersen et al. 1964). A limitation common to these

areas of interest is that they focus on large-scale changes affecting most if not all of the brain at once. In particular, some epileptic seizures, like primary generalized tonic-clonic seizures, cannot be reliably traced back to a specific location inside the brain (Blumenfeld and Taylor 2003). This limits the amount of information we can currently obtain from canine EEG.

1.3 The sleep spindle

Sleep spindles are relatively brief (lasting 0.5-5 seconds), bursts of rhythmic activity, observed predominantly in the non-REM EEG signal (Jankel and Niedermeyer 1985; De Gennaro and Ferrara 2003). They have been extensively studied with regard to their mechanistic underpinnings in the cat (Steriade et al. 1985; Bazhenov et al. 1999; Steriade and Timofeev 2001) and rat (Kandel and Buzsáki 1997; Meeren et al. 2009; Sitnikova 2010), which makes them ideal candidates for increasing the information content of non-invasive EEG studies, provided we can be certain these oscillations are analogous between species[6]. Another advantage is the wide range of functions associated with the occurrence, amplitude and oscillation speed, i.e. frequency (waves/second), of the spindling bursts. In humans, they have been implicated in sleep stability (Dang-Vu et al. 2010), memory consolidation, mostly measured as post-sleep recall of novel information (Gais et al. 2002; Clemens et al. 2005, 2006; Cox et al. 2012; Lustenberger et al. 2016), and even general mental ability (Schabus et al. 2006; Ujma et al. 2014; Ujma 2018). Changes in spindling features also accompany various psychiatric conditions (Ferrarelli et al. 2007; Wamsley et al. 2012; Plante et al. 2013; Latreille et al. 2015), the trajectories of early development (Bódizs et al. 2014; Hahn et al. 2018), and aging (Guazzelli et al. 1986; Crowley et al. 2002; Martin et al. 2012), as well as pathological aging in particular (Smirne et al. 1977; Ktonas et al. 2007; Rauchs et al. 2008; Latreille et al. 2015).

These relationships will be elaborated in more detail in the next chapters. Important for now is that, in spite of their implication in many interesting and relevant functions of the brain, our knowledge of how universal spindles are in the animal kingdom is extremely limited. Although described in a range of mammalian species (Kryger et al. 2011) sleep spindles are not confirmed beyond face value outside of cats, rodents and humans. Any evidence for analogue mechanism or similar functional associations (even just by correlation) are limited to these three clades. The question of how universal spindling is, is not trivial. Several studies support the notion that avian brains do not display, nor require, mammal-like spindles (Rattenborg and

[6] See discussion of face-, predictive- and construct validity later in this chapter.

Martinez-Gonzalez 2011; Rattenborg et al. 2011; Van Der Meij et al. 2019). For this reason, in addition to increasing the informational value of canine EEG, a focus on canine sleep spindles will also contribute to questions of brain evolution.

To determine, however, if EEG signals that appear similar between species are also correctly identified as analogues in the functional and evolutionary sense, it can be useful to adopt a version of the validation system discussed with regard to animal models of (psycho)pathology (Overall 2000; Coenen and van Luijtelaar 2003; van der Staay et al. 2009; Nestler and Hyman 2010; Vervliet and Raes 2013; Topál et al. 2019). In particular, three types of validity are repeatedly discussed in the field of clinical animal models: ***face validity***, ***predictive validity*** and ***construct validity*** (but see van der Staay et al. (2009) for an additional discussion of external validity). These validities are usually defined relative to a disease model, but I will discuss how the underlying thought can be applied more generally to analogies between species. Moreover, I will organize our further introduction into sleep spindles based on the three validities.

Face validity. An animal model of disease fulfills the criterion of face validity if the signs and symptoms are phenotypically similar as in the condition that a researcher attempts to model. This more broadly includes anything that can be directly observed, not only behaviorally, but also physiologically (such as anatomical and chemical changes; see Nestler and Hyman (2010) for an in-depth review). Importantly, in EEG studies face validity can also refer to the appearance of EEG signals. For example, Coenen and Van Luijtelaar (2003) describe that in both rats and humans absence seizures are characterized by a series of spikes and waves in the EEG. I therefore will introduce the important parameters that were used in the past for the visual detection of spindles in association with the concept of face validity.

Predictive validity. An animal model of disease fulfills the criterion of predictive validity if a treatment/intervention produces the same or similar result to what we would predict based on our experience with the disease in humans. While usually defined narrowly with regard to pharmacological interventions in the context of disease models, the underlying rationale is that similar responses to medication suppose similar causal dynamics between model and modeled disease. Applying this idea outside the context of disease could be done by comparing similar changes between supposedly analogue systems in response to the same treatment. In the context of sleep spindles specifically this means that I will define "treatment" more broadly, and I will look at work which suggests that the human sleep spindle responds to "treatments" such as the hormonal changes of the menstrual cycle or increased exposure to novel

information prior sleep. These "treatments" can also be used to argue for similar causal dynamics. Such a broader interpretation of predictive validity is also described in van der Staay et al. (2009), although most authors, including him, agree that in the biomedical sciences a narrow definition concerning responses to medication or therapy is more commonly accepted. The contents discussed here under the label "predictive validity" might overlap even more closely with van der Staay's "external validity" which concerns analogies between species more generally, as they support the generalizability of the model.

Construct validity. An animal model of disease fulfills the criterion of construct validity if the causes of the symptoms (e.g. mutations, lesions) are applicable both in the model and in the modeled disease. Outside the context of disease I will use this to mean shared mechanisms, i.e. similarity in the underlying mechanisms. We will discuss the causal dynamics that lead to sleep spindles in the EEG signal in association with the concept of construct validity. This broader definition of construct validity is also supported by van der Staay et al. (2009).

1.3.1 Face validity: What spindles look like in EEG

Sleep spindles were discovered in the early days of sleep physiology (Berger 1933; Loomis et al. 1935). This is likely due to defining characteristics that make most spindle events easy to detect by eye without extensive data manipulation. To the present day some authors evaluate automatic detection algorithms for sleep spindles by comparing their performance to that of expert scorers (Nonclercq et al. 2013), with some also arguing that visual detection is still more reliable (O'Reilly and Nielsen 2015). While introducing the defining characteristics of spindles, we will for now leave these assumptions unquestioned, but revisit them in our later discussion on spindle detection methods.

In some rare cases, the shape of an EEG event is its most defining feature. This is true for spike-wave discharges, the EEG correlates of absence seizures, which can be recognized as an alternating pattern of sharp spikes and round waves (Coenen and van Luijtelaar 2003) or K-complexes, which are characterized by a single, large amplitude event which extends below and above the baseline EEG trace and is asymmetric around its polarity reversal point (Bremer et al. 1970). A set of shape-defining features for sleep spindles were early on proposed by Dutertre (1977) who states that "spindle waves are monomorphic, diphasic and symmetrical with respect to the baseline" (cited in Jankel and Niedermeyer (1985)). This description means that spindles appear as a collection of sinusoidal waves, each of which is roughly equal in size, shape and duration. However, the assertion that the amplitude of the entire train of waves is rising and falling from the beginning to the end of the spindling event (and which gave this

EEG signature its name) is according to Jankel and Niedermeyer (1985) "the exception rather than the rule".

In short, shape is not a very helpful criterion to recognize sleep spindles[7]. All rhythmic and synchronous brain activity will appear as a train of waves in the EEG signal, including alpha, theta and delta waves (Steriade and Llinás 1988). The distinguishing features between different types of rhythmic synchronization are their duration, amplitude (size) and frequency (waves/second).

Duration is a clear, though not sufficient, distinguishing feature. With an average duration of 1-6 seconds (Dutertre 1977), and a minimum of 0.5 seconds (Rechtschaffen and Kales 1968), sleep spindles are noticeably shorter than other bouts of synchronized activity like alpha or delta waves (Steriade and Llinás 1988).

The estimated minimum amplitude of spindles varies from 8 to 25 μV (Fish et al. 1988; Zeitlhofer et al. 1997). An absolute threshold value is problematic, however, because individual differences in skull thickness can result in different EEG amplitudes. Instead, Nonclercq et al. (2013) propose to only consider events with an amplitude a standard deviation above the filtered[8] signal's average as potential sleep-spindles.

Frequency is the most crucial feature for telling apart different synchronized patterns in the EEG signal. Although the proposed frequency range of sleep spindles varies somewhat in humans (Jankel and Niedermeyer 1985; De Gennaro and Ferrara 2003) and a lot across species (Kryger et al. 2011), it is always estimated notably higher than the Delta waves (1-4 Hz in humans, 2-4 Hz in rodents) and slow oscillations (0.5-1 Hz) that otherwise predominate during non-REM sleep[9] (See Figure 3 for a typical example of how spindles appear against the background activity characterizing non-REM sleep). In humans, the definition of the frequency range of spindles, also known as the "sigma" range (Jankel and Niedermeyer 1985), varies between authors mostly with regard to the lower boundary which some estimate as low as 8 or 9 Hz (De Gennaro and Ferrara 2003; De Gennaro et al. 2008; Bódizs et al. 2009), others at 11 Hz (Clemens et al. 2005; Cox et al. 2012) and some as high as 12 Hz (Uchida et al. 1994; Nobili

[7] Although many spindles are readily visible to human scorers this is not due to their shape.
[8] Because spindles 'swim' on top of larger waves (see part 1.1.1 in the introduction about the characteristics of non-REM sleep) these slower activity is usually filtered out to enable measuring amplitude relative to baseline.
[9] It should be noted that sleep spindles are particularly abundant in humans during sleep stage 2 of the non-REM sleep phase, however in rats and dogs (Genzel et al. 2014; Kis et al. 2014) non-REM sleep is seldomly divided in sub-categories, making this observation redundant knowledge in attempting to compare spindle occurrence between humans and other mammals.

et al. 1999; Genzel et al. 2014). Notably, most authors agree that 16 Hz is the upper boundary for sigma (Uchida et al. 1994; Nobili et al. 1999; Clemens et al. 2005; Bódizs et al. 2009; Cox et al. 2012; Genzel et al. 2014). Rarely spindling frequency is also narrowly defined between 12 and 14 Hz (Rechtschaffen and Kales 1968; Nonclercq et al. 2013), which I will refer to later on as the "strict" definition of sigma.

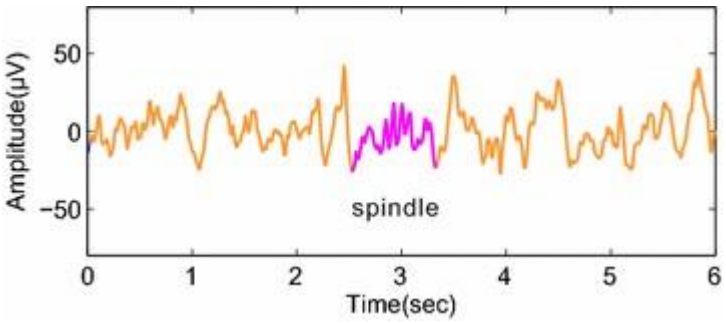


Figure 3. Spindles appear as brief (0.5-6 seconds) bursts of high frequency wave-trains (purple) against the background of larger and slower waves (orange) defining non-REM sleep. Adopted from Zhuang, Li and Peng (2016). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

Frequency is also a crucial criterion for dividing spindles into subtypes. Initially Gibbs and Gibbs attempted to distinguish three types (1950) – a 10 Hz, 12 Hz and 14 Hz spindle, but a dichotomy between 'slow' and 'fast' spindles became the most widely accepted subdivision (Jankel and Niedermeyer 1985). In this context "slow" refers to a low frequency and "fast" to a high frequency. This dichotomy is not an arbitrary distinction, but based on the observation that in humans (Gibbs and Gibbs 1950, 1961) and rodents (Terrier and Gottesmann 1978) spindles expressed in central and posterior parts of the cortex are of higher frequency. More precisely, the frequency of slow and fast spindles overlap between 12.5 and 13 Hz (Gibbs and Gibbs 1950), which is operationalized by many authors as handling a cut-off value of 13 Hz to categorize spindle detections into slow ($\leq$ 13 Hz) and fast ($\geq$ 13 Hz) (Schabus et al. 2006, 2007; Ackermann et al. 2015; Hennies et al. 2016; Hahn et al. 2018). Importantly, slow and fast spindles also differ in their pharmacological response profile (Ayoub et al. 2013), their developmental trajectory (Hahn et al. 2018), their response to sexual hormones (Bódizs 2017) and which types of learning and memory they appear to support (Schabus et al. 2008). All of these differences will be discussed in more detail in the next section.

1.3.2 Predictive/External validity: What predicts spindle expression and what spindles predict

As discussed earlier, in the context of disease models, predictive validity is very narrowly defined as analogous pharmacological responses between model and modelled disease. If we want to evaluate the dog as a model animal for studying sleep spindle activity, which is,

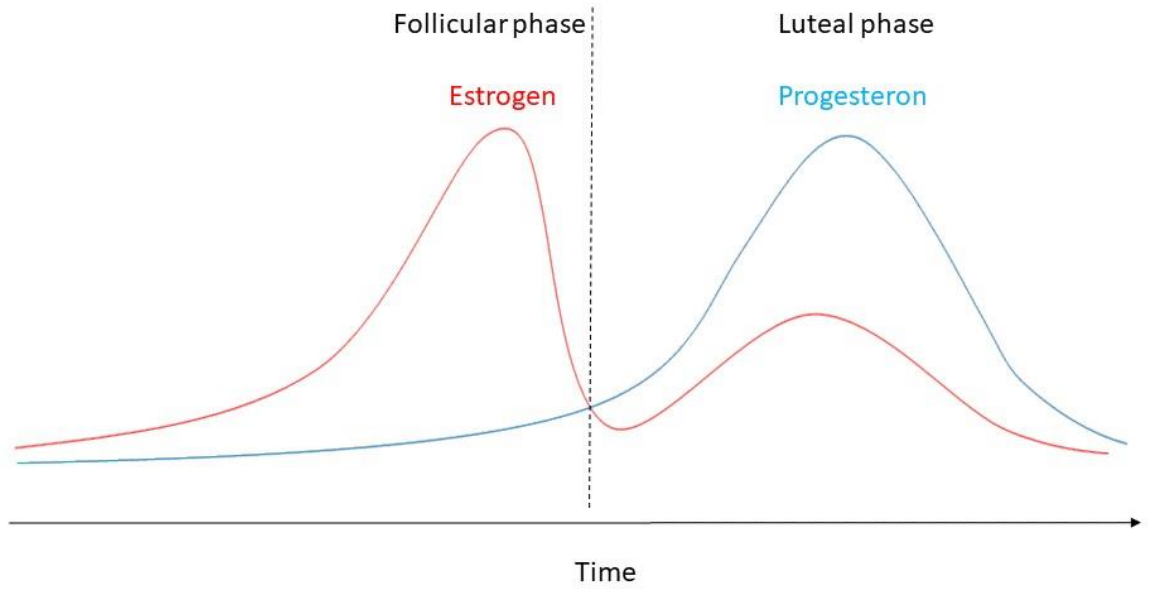
however, not a disease, we have to define predictive validity in a broader sense. The choice to handle a broad definition is also supported by van der Staay's description of the validities and concerns a zone of overlap between the concepts of predictive and external validity (van der Staay et al. 2009). As yet, I will begin this chapter by outlining some of the known associations between spindle expression and pharmacological intervention, following thus the narrow definition at first. This will include the pharmacological dynamics of neurotransmitters and hormones.

Ayoub and colleagues (2013) studied the effects of Carbamazepine and Flunarizine on slow and fast spindles, as well as slow oscillations in human subjects. Carbamazepine is a sodium (Na+) channel blocker (Rogawski et al. 2016), in particular voltage-sensitive channels[10] which help translate EPSPs into action potentials, hence it is useful in treating epilepsy and schizophrenia, which are characterized by excessive excitability of cortical neurons (Badawy et al. 2007; Badawy and Jackson 2012; Hasan 2013; Lakatos et al. 2013). Ayoub et al. (2013) found that administering carbamazepine increased the power[11] of slow spindles and slow oscillations, in particular increasing the absolute occurrence of slow spindles, but the same drug reduced the amplitude and occurrence of fast spindles. Flunarizine, a calcium (Ca+) blocker[12] (Peters et al. 1991) also reduced fast spindle occurrence, but slow spindles and slow oscillations remained unaltered. From earlier studies in cats (Ganes and Andersen 1975; Ganes 1976) and rats (van Luijtelaar 1997) it is also known that benzodiazepines and barbiturates, both GABA agonists and central nervous system depressants (Stahl 2000) enhance spindle expression. Jankel and Niedermeyer (1985), however, have questioned to what degree these anesthesia induced spindles are related and comparable to the spontaneous sleep spindle observed in natural sleep.

Other work with human subjects suggests that sexual hormones, too, have a pharmacological effect on spindle expression (Gaillard and Blois 1981; Driver et al. 1996; Huupponen et al. 2002; Baker et al. 2007; Genzel et al. 2012; Ujma et al. 2014; De Zambotti et al. 2015; Bódizs 2017; Sattari et al. 2017). It is currently not clear if women generally display more (Gaillard and Blois 1981; Ackermann et al. 2015), or less (Ujma et al. 2014; Bódizs 2017) sleep spindles

[10] See early introduction (part 1.1) about the role of voltage sensitive sodium/natrium channels in the generation of EPSPs and action potentials.
[11] Power is a common variable in EEG research, represents the squared amplitude of a frequency component in the signal.
[12] The possible role of calcium will be discussed in the next chapter (1.2.3), when we turn to the underlying mechanisms of spindling.

than men. Part of this contradiction might be due to localized and hemispheric differences between men and women (Huupponen et al. 2002; Bódizs 2017) (e.g. higher percentage of spindles detected over the left frontal cortex in women). A more relevant finding, however, was that spindle density (occurrence/minute), duration and frequency vary with the menstrual cycle. An increase of fast spindle occurrence was reported several times in the luteal phase of the cycle (Driver et al. 1996; Baker et al. 2007; De Zambotti et al. 2015). However, Bódizs (2017), using data from invasive recordings in volunteering patients did not confirm a rise in fast spindle occurrence, but rather an increase in spindling frequency, also at the peak of the luteal phase. The invasive data also confirmed that women have higher fast spindle amplitudes (previously observed in Ujma et al. (2014)) and that these are not artefacts caused by differences in skull thickness. While estrogen peaks twice during the menstrual cycle, progesterone peaks uniquely during the luteal phase (Baker and Driver (2007), also see Figure 4), suggesting that higher fast spindle occurrence/frequency[13] is specifically the result of high progesterone levels. Progesterone, its synthetic versions, and in particular its metabolites, are in turn GABA agonists[14] (Harrison and Simmonds 1984; Majewska et al. 1986; Wu et al. 1990; Bitran et al. 1993) and GABA plays a crucial role in the generation of spindle oscillations (Steriade and Llinás 1988; van Luijtelaar 1997).



[13] An increase in spindle frequency and an increase in fast spindle occurrence might actually be the same effect, depending on how the researcher delineates slow and fast spindles.

[14] GABA (gamma-amino butyric acid) is an inhibitory neuro-transmitter, this means its action reduces the chance of a neural cell to reach the threshold for firing action potentials. A GABA agonist is a substance that enhances GABA or its effects on neural cells (see also Stahl (2000)).

Figure 4. Relative changes in concentration of estrogen and progesterone throughout the follicular and luteal phases of the menstrual cycle.

Extending beyond the pharmacological, a "treatment" which increases spindle occurrence reliably in both humans (Gais et al. 2002; Fogel and Smith 2006; Schmidt et al. 2006; Schabus et al. 2008; Mölle et al. 2009) and rats (Eschenko et al. 2006; Mölle et al. 2009) is exposure to novel information prior to sleep, tied to the incentive or instruction to engage in learning, which is usually demonstrated in contrast to an otherwise identical control condition which requires the same work-load but provides no new information nor instructions to learn. Another event predicting spindle behavior, which also connects to learning, are hippocampal ripples - an electrophysiological pattern associated with hippocampal replay of previously experienced information (mostly trajectories, in a maze, run by mice or rats (Jadhav and Frank 2014)). Sleep spindles were found to be phase-locked to ripples in humans (Clemens et al. 2011) and the two were temporally correlated in rats (Siapas and Wilson 1998). Importantly, ripples cannot be observed in surface EEG (Clemens et al. (2011) used invasive recordings in volunteering epilepsy patients), hence spindle occurrence can be used to infer heightened hippocampal replay during sleep.

Spindle occurrence in turn is a predictor of learning performance when measured during an episode of sleep placed between acquisition and recall testing (Gais et al. 2002; Clemens et al. 2005, 2006; Schmidt et al. 2006; Tamaki et al. 2009; Barakat et al. 2011; Seeck-Hirschner et al. 2012; Cox et al. 2012; Astill et al. 2014; Gruber et al. 2015; Hennies et al. 2016; Lustenberger et al. 2016; Yordanova et al. 2017; Kuula et al. 2019). Amplitude, frequency or duration have also been shown to predict learning performance (Schabus et al. 2008; Tamaki et al. 2009; Kuula et al. 2019). The relationship of learning to occurrence is linear and positive: the more spindles, the better the post-sleep recall (when amplitude, frequency and duration are investigated: larger amplitudes, higher frequencies and longer durations). This does not seem to depend on whether one uses absolute numbers (Clemens et al. 2005, 2006; Seeck-Hirschner et al. 2012) or density, i.e. spindles/minute (Gais et al. 2002; Barakat et al. 2011; Cox et al. 2012; Seeck-Hirschner et al. 2012; Gruber et al. 2015; Hennies et al. 2016; Lustenberger et al. 2016; Kuula et al. 2019). The effect is found both when spindles are distinguished into fast and slow (Tamaki et al. 2009; Barakat et al. 2011; Gruber et al. 2015; Lustenberger et al. 2016; Kuula et al. 2019) and when occurrence is calculated for all detections in the sigma range without distinction (Gais et al. 2002; Clemens et al. 2005, 2006; Cox et al. 2012; Seeck-Hirschner et al. 2012). Intriguingly, when a distinction is made, many authors report that only

fast spindles predict learning (Tamaki et al. 2009; Barakat et al. 2011; Astill et al. 2014; Gruber et al. 2015; Lustenberger et al. 2016; Yordanova et al. 2017; Hahn et al. 2018), but this might depend on the type of information to be learned. For most studies reporting an exclusive involvement of fast spindles, the experimental set-up revolves around learning a motor sequence (Tamaki et al. 2009; Barakat et al. 2011; Astill et al. 2014; Lustenberger et al. 2016; Yordanova et al. 2017). Concerning the possible role of slow-spindles the answer is accumulating more slowly and indirectly. Clemens et al. (2005), for instance, found an association between recall success and spindle occurrence recorded over the frontal cortex, where slow spindles predominate, however these authors did not distinguish fast and slow spindles. Kuula et al. (2019) found a negative correlation between false memories and slow spindle density, but only for slow spindles recorded over the central midline electrode (Cz) and only in adolescent girls. Schabus et al. (2008) found an increase of frontal slow spindles after subjects engaged in learning, but only in "highly gifted" individuals (identified by their relative performance on the Advanced Progressive Matrices within the sample), whereas Schmidt et al. (2006) found increases in slow spindle occurrence only when subjects had to encode more abstract information. What is universally shared between these reports of slow spindle associations with learning and memory is the use of verbal material (word-pair association tasks) to measure learning, but see Gruber et al. (2015) for an exception where verbal learning is predicted exclusively by fast spindles. In short: there is more evidence for fast spindle involvement in learning, however it is predominantly demonstrated for motor and sensorimotor learning. Meanwhile, there is also indirect yet slowly converging evidence towards a possible slow spindle involvement more exclusive to verbal learning. It is also conceivable that some systematic difference in the structure of most motor learning tasks, distinct from most verbal learning tasks might be the underlying difference between studies that find fast or slow spindle involvement in learning, rather than the actual learning material itself. The works of Schabus et al. (2008) and Schmidt et al. (2006) for instance converge on the notion that the difficulty of the memory task might play a role and that slow spindles selectively support the encoding of more demanding associations (operationalized by Schmidt and colleagues as more abstract word-pairs). It is unlikely that the distinction of function is literally about words or movements (see exceptions like Gruber and colleagues (2015)). A very few studies in which fast and slow spindles were distinguished found both (Hennies et al. 2016) or none (Ackermann et al. 2015) to predict recall performance.

The negative result of Ackermann et al. (2015) is an alarming note of caution against a strong link between sleep spindles and learning, due to the large sample size on which it is based (N = 929). However, work with animal models has simultaneously revealed mechanisms that potentially explain why an association is as yet often observed (Rosanova and Ulrich 2005; Latchoumane et al. 2017). A possible explanation for this controversy is provided by studies that aim to specify the exact conditions under which sleep spindles predict learning. These include the timing of spindles within non-REM sleep (Cox et al. 2012) and relative to the up-states of slow oscillations (Clemens et al. 2011; Latchoumane et al. 2017)[15], but also work that suggests spindles are specifically involved in the enhancement of schema-conform memories (Hennies et al. 2016; Latchoumane et al. 2017).

A final aspect to discuss with regard to spindle-learning associations is a body of work suggesting that they depend on when sleep spindles occur in the sleep cycle and relative to slow oscillations, at least in humans. Sleep spindles are generally most abundant throughout non-REM sleep and were first discovered in stage 2 of the 4 stages of non-REM, during which they are most visible (Jankel and Niedermeyer 1985; De Gennaro and Ferrara 2003). Work by Cox et al. (2012) suggest, however, that spindle density only predicts learning when detections are selected for occurrence during Slow Wave Sleep (SWS). In humans SWS refers to stages 3 and 4 of non-REM, and usually excludes stage 2. Many studies reporting spindle-learning associations either do not control for the exact sleep stage (Clemens et al. 2005) or focus on stage 2 instead (Seeck-Hirschner et al. 2012; Hennies et al. 2016; Kuula et al. 2019). However, work by Mölle et al. (2009) suggests that timing relative to slow oscillations is more relevant, which might explain the apparent contradiction in the literature. While slow oscillations happen throughout non-REM, they are most abundant in stages 3 and 4 (thus SWS). In particular, Mölle and colleagues observed that ripples, linked to hippocampal replay, and spindles tend to co-occur mostly at the so-called up-states of slow oscillations[16]. Findings of these kind may as yet have no relevance for human-animal comparisons. First, most animal studies do not distinguish different stages of non-REM sleep (Genzel et al. 2014), and it is also not recommended for dogs, as their EEG signal does not support the existence of separate non-REM stages (Kis et al. 2014). Second, while Mölle et al. (2009) found prior learning to increase both ripple/spindle occurrence and synchronization with slow oscillations in humans, only

[15] The issue of sleep spindle timing is discussed in more detail throughout the next paragraph.
[16] In the literature on sleep-dependent memory consolidation the peaks and valleys of slow oscillations are called up-states and down-states.

unsynchronized learning-induced ripple/spindle increases were observed in rats (but see Latchoumane et al. (2017) for more human-like synchronization in mice). This raises the question if all animals require the synchronization between spindles, ripples and slow oscillations we observe in humans.

Another measure of cognitive performance researchers often find to be predicted by spindle activity is general mental ability or IQ (Bódizs et al. 2005, 2014; Schabus et al. 2006, 2008; Ujma et al. 2014, 2016). A recent meta-analysis suggests that IQ is most reliably predicted by both slow and fast spindle amplitudes, as well as slow spindle duration (Ujma 2018), but the described effect sizes are modest and thus likely reflect an indirect association[17]. Occasionally researchers report that IQ only correlates positively with spindle activity in women (Ujma et al. 2014, 2016).

High levels of spontaneous sleep spindle occurrence were also observed to predict sleep-stability in the face of noise (Dang-Vu et al. 2010) and would make sense given the state of low transmission of information between thalamus and cortex during a spindling event (Steriade and Llinás 1988). It is, however, not always a straightforward relationship: in the mid-luteal phase of their cycle, for instance, women display both more awakenings and more fast spindles (De Zambotti et al. 2015), while the reduction of spindle occurrence also does not predict an increase in awakenings (Feinberg et al. 1967; Guazzelli et al. 1986).

Changes in all spindle characteristics can be observed with regard to development, age and pathological aging. In early childhood a mild positive correlation is observed between age and spindle amplitude (Ujma et al. 2016). From childhood to adolescence the occurrence of fast spindles increases in central and parietal parts of the cortex, while initially slow spindles predominate on all measured locations (Hahn et al. 2018). The literature on normal and pathological aging is more abundant. Sleep spindles are of interest to the study of cognitive aging due to their association with memory, learning and IQ, all of which decline in the elderly (Thomas et al. 2007; Brickman and Stern 2010; McQuail et al. 2015). Guazzelli et al. (1986), however, did not find spindle characteristics to directly predict the level of cognitive functioning in a large sample of ”normally” aging subjects (N = 48). Instead, it is possible to distinguish or predict Alzheimer and other types of dementia from normal aging using spindle measures (Smirne et al. 1977; Ktonas et al. 2007; Rauchs et al. 2008; Latreille et al. 2015;

[17] For instance, sleep spindle amplitude and duration, as well as IQ, decline with age and in many pathological conditions (see also pages 26, 27).

Gorgoni et al. 2016). In Alzheimer's disease and Pick's disease sleep spindles can be entirely absent (Smirne et al. 1977). Other consistent, yet subtler changes that characterize both normal and pathological aging (in comparison with younger subjects) are a decline in occurrence (Smirne et al. 1977; Guazzelli et al. 1986; Crowley et al. 2002; Rauchs et al. 2008; Martin et al. 2012; Latreille et al. 2015; Gorgoni et al. 2016), which in Alzheimer patients that still express spindles is more pronounced for the fast spindle type (Rauchs et al. 2008; Gorgoni et al. 2016), and a decline in amplitude (Guazzelli et al. 1986; Landolt and Borbély 2001; Martin et al. 2012; Latreille et al. 2015). An increase in spindle frequency among the elderly has also been reported (Principe and Smith 1982; Crowley et al. 2002; Ktonas et al. 2007) and validated as a biomarker for dementia (Ktonas et al. 2007).

Lastly, spindles appear to be promising biomarkers of various psychiatric conditions as well, such as schizophrenia, depression and ADHD (Ferrarelli et al. 2007; Lopez and Hoffmann 2010; Plante et al. 2013; De Dea et al. 2018; Merikanto et al. 2019). Most of these studies report reduced spindle occurrence and/or amplitude, as we observe in aging (Ferrarelli et al. 2007; Lopez and Hoffmann 2010; Merikanto et al. 2019). One exception is a study by Plante et al. (2013), which found greater density, amplitude and duration for sleep spindles in female patients with major depressive disorder compared to controls.

1.3.3 Construct validity: How are spindles created in the brain

In the context of disease models, construct validity refers to the origin of the disease, i.e. a high construct validity is provided, for instance, if the model shares a comparable mutation to one that is responsible for the disorder. Since my goal will be to evaluate a potential model animal for studying sleep spindles, this question can be modified to ”Are canine spindles the result of the same neural mechanisms that underlie spindling in humans and other commonly studied species?”.

The thalamus (Steriade and Llinás 1988) and cortex (Kandel and Buzsáki 1997; Rosanova and Ulrich 2005; Sitnikova 2010) each contribute to the generation of sleep spindles and the shaping of their visible features. The process is usually described as a cascade of events beginning in the thalamus, eventually activating local micro-circuits in the cortex. Although the thalamic dynamics preceding spindles are themselves modulated by signals from the brainstem and cortex (Steriade and Llinás 1988), this narrative does not merely serve a simplified understanding, but is based on a series of studies showing that the thalamus (Morison and Bassett 1945) and more specifically the reticular thalamic nucleus (RTN) (Steriade et al.

1985; Bazhenov et al. 1999) are sufficient to produce oscillations in the sigma range when isolated from other parts of the brain in cats.

To better understand the different mechanisms leading to spindles and the shaping of their features, we should first take a simplified schematic look (Figure 5) at the thalamo-cortical system. The cortex is the evolutionarily youngest part of the brain, forming the outermost layer of the mammalian brain, and is associated with abstract (motor) planning and impulse control (frontal and premotor cortices), the integration of sensory information into multi-modal and abstract categories (temporal and parietal lobes), and the storage of long-term memory (entire cortex) (Kolb and Whishaw 2001). Most 'raw' sensory information is relayed to the cortex via the thalamus, where ascending signals, grouped by the sensory domain to which they belong (i.e. visual, auditory etc.) can be filtered or blocked (Purpura 1970). Hence, the thalamus is often referred to as a 'gatekeeper' or a place of 'gating' (McCormick and Bal 1994; Moustafa et al. 2017). Descending motor commands can also be blocked at the level of the thalamus (Crabtree and Isaac 2002) and from here it seems that general responsiveness to the environment, characterizing different vigilance states, is also controlled (Steriade et al. 1969; Steriade and Llinás 1988). These gating functions have been more specifically narrowed down to the RTN (Steriade et al. 1985). To understand how exactly the RTN does its work and what this has to do with spindles, we need to revisit, and add to, some of the fundamental physiology discussed in the very beginning of the introduction.

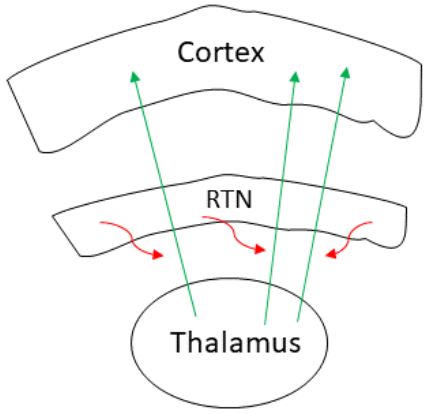


Figure 5. A schematic representation of the spatial arrangement between cortex (outermost layer of the brain), thalamus and the reticular complex, also known as the reticular thalamic nucleus (RTN, sometimes also abbreviated as TRN or RE). The RTN sends out inhibitory projections (red arrows) to thalamo-cortical relay cells (green arrows).

Neurons have to fire an action potential to affect other neurons, and action potentials are triggered by changes in voltage, from more negative (typically around -70 mV difference intracellular to extracellular space) to more positive, with action potentials being generated when a threshold is reached at ca. -40 mV. This is true for many cells and the standard example used in textbooks (Kolb and Whishaw 2001): the mechanism relies on voltage-sensitive Na+ channels, which open at this threshold and change the intracellular voltage to even more positive values (ca. +20 mV during the actual action potential, Figure 1). For this reason, synaptic transmissions which increase the positively charged sodium concentration in post-synaptic cells are called EPSPs i.e. excitatory post-synaptic potentials, since they increase the likelihood of action potentials in the post-synaptic cell. Meanwhile, signals that increase the intracellular occurrence of negatively charged chloride and/or decrease the concentration of K+, and thus reduce the chance of an action potential are called IPSPs (inhibitory post-synaptic potentials). Finally, we should recall, that the process of voltage-change towards more positive values and thus towards action potential generation is called depolarization, while increased negative values of intracellular voltage, associated with lowered likelihoods of action potential generation are called hyperpolarization. To get a good picture of the spindle-relevant mechanisms, we should add a complication not discussed in entry level neuroscience: the existence of low-threshold calcium channels (permissive to the positively charged Ca^{++}), characterizing both thalamo-cortical relay cells and cells in the RTN (Bazhenov et al. 1999; Destexhe and Sejnowski 2002). Unlike the earlier described natrium/sodium channels, these voltage sensitive calcium channels are triggered by a threshold value below the resting potential of -70 mV; thus hyperpolarization can trigger action potentials in these cells, due to the influx of the positively charged calcium. Neural activity triggered by IPSPs and hyperpolarization in this way is known as ‘rebound’ activity.

Steriade and Llinás (1988) provide a comprehensive overview of the history of research on thalamic mechanisms and their relationship to sleep spindles. I will skip much of this discussion, as it is outside the scope of the thesis. Importantly, Steriade and colleagues themselves demonstrated two important relationships for the mechanistic understanding of spindles. First, they showed that in the cat thalamic synchronization in the 7-14 Hz range (feline sigma), beginning in drowsiness and continuing throughout non-REM sleep, is associated with decreased transmission of information from subcortical to cortical brain areas via the thalamus (Steriade et al. 1969, 1971; Glenn and Steriade 1982). Second, again in the cat, they reported a disruption of 7-14 Hz synchronization in thalamic neurons disconnected from the RTN

(Steriade et al. 1985) and a sustained 7-14 Hz rhythmicity in completely isolated RTN preparations (Bazhenov et al. 1999).

RTN neurons are GABAergic (Houser et al. 1980; Hendrickson et al. 1983; Gabbott et al. 1985), meaning they release the neurotransmitter GABA when firing, which induces IPSPs in post-synaptic neurons by opening channels for the negatively charged Cl^- (Stahl 2000; Kolb and Whishaw 2001). RTN neurons hyperpolarize each other (Bazhenov et al. 1999), as well as thalamo-cortical relay cells (Scheibel and Scheibel 1966; Jones 1975). In the awake state, RTN neurons presumably block each other from projecting to thalamic relay cells because of this organization. However, additional hyperpolarizing inputs onto the RTN during non-REM sleep increase this hyperpolarization until the threshold of low-threshold activated calcium channels is reached (Bazhenov et al. 1999), which results in rebound net excitation. The activated RTN neurons hyperpolarize thalamic relay cells, which on one hand reduces their excitability to inputs from outside the thalamus (Steriade et al. 1969, 1971; Glenn and Steriade 1982), explaining the reduced thalamo-cortical transmission, and on the other activates the low-threshold calcium channels of thalamo-cortical relay cells (Destexhe and Sejnowski 2002). The former hypothetically explains why in some studies we see a relationship between spindle occurrence and sleep stability (Dang-Vu et al. 2010), and why some researchers propose that spindles support memory consolidation by protecting memories from interference (Genzel et al. 2014). This in turn aligns with experiments in which spindles specifically predict resilience to false memories (Kuula et al. 2019) and also with the observation that in awake rats memory replay is accompanied by suppression of thalamo-cortical transmission, as well (Yang et al. 2019).

The observation that the hyperpolarized thalamo-cortical cells respond with rebound-bursts of activation (Destexhe and Sejnowski 2002) explains the cortical involvement in the expression of spindles (Kandel and Buzsáki 1997; Rosanova and Ulrich 2005; Sitnikova 2010; Peyrache et al. 2011) and spindle frequency (waves/second observed for a single spindle event), which according to Steriade and Llinás (1988) is the result of alternating RTN-induced inhibition and rebound activation. In particular, during non-REM sleep, the RTN releases bursts of IPSPs onto the thalamic relay cells every 3-10 seconds (Steriade et al. 1987; Steriade and Llinás 1988). The bursts of IPSPs last about 70-150 milliseconds and their exact duration predicts what frequency within the sigma range a spindle will exhibit and is itself predicted by the overall level of inhibition in the brain – with which Steriade and Llinás (1988) explain the general observation of lower spindle frequencies under deep anesthesia (see also Jankel and

Niedermeyer (1985)). The latter hints at a possible explanation for the slow versus fast spindle dichotomy.

Slow spindles are observed predominantly over the frontal cortex in both humans and rats (Gibbs and Gibbs 1961; Terrier and Gottesmann 1978), which is also the area most inhibited during spindles in the rat (Peyrache et al. 2011). Indeed both empirical and theoretical efforts (Sitnikova 2010; Peyrache et al. 2011) support the notion that the rebound signals coming from hyperpolarized thalamic relay cells, by activating local inhibitory inter-neurons, isolate the cortex from hippocampal inputs, in addition to the reduced thalamo-cortical transmission at the initiation of a sleep spindle. This cortical inhibition, in turn, is assumed to explain another characteristic of how spindles appear in EEG signals – more inhibition accounts for larger amplitudes, according to one computational model (Sitnikova 2010). Finally, to conclude on the cortical components of sleep spindle activity, Rosanova and Ulrich (2005) found evidence that sleep spindles might[18] induce long-term potentiation (LTP) in the cortex, which is considered a candidate mechanism for associative learning (Lynch 2004). This would mean that the role of spindles in learning might extend beyond shielding memories from interference with noise, and studies showing that treatments increasing spindle activity can improve learning performance (Lustenberger et al. 2016) seem to support this.

1.4 Modelling sleep spindles in animals

In this last part of the introduction, we are closing in on the evaluation of dogs as model animals for comparative research on sleep spindles. To do so, we will first have a look at existing comparative research and model animals, their advantages and shortcomings, then turn to the state-of-the-art knowledge about sleep spindles in dogs and formulate, finally, how I conducted this investigation.

1.4.1 Evaluating model animals in sleep spindle research, based on the three validities

As hinted earlier, for most mammalian species where sleep spindles have been observed (Kryger et al. 2011), only face validity was used as a criterion. Thus, we actually do not know if real spindles are exhibited in most mammals, as it has not been confirmed whether the similar looking EEG patterns are indeed caused by the same mechanisms or involved in the same causal dynamics (e.g. predictive relationships with learning). Considering the wide range of defining frequencies claimed for sleep spindles of different species, even face validity is not strong. Taking into account that birds and reptiles do not exhibit spindles (Rattenborg and

[18] We say „might" because the study used an in-vitro preparation and a simulation of spindle-like firing patterns, modelling cat spindles onto cells derived from rat brains.

Martinez-Gonzalez 2011; Rattenborg et al. 2011; Shein-Idelson et al. 2016; Van Der Meij et al. 2019) it is also not certain that they are a universal feature of mammalian sleep.

A historically promising model animal in sleep research – the cat (Pampiglione 1971), shows strong construct validity. In fact, most of what we know about the mechanisms underlying sleep spindles was discovered in this species first (Steriade and Llinás 1988) and there is indirect evidence[19] that it applies to human sleep spindles as well (Schabus et al. 2007). In the cat, however, the broad definition of predictive validity and external validity (van der Staay et al. 2009) have not been evaluated. No studies have investigated whether in cats sleep spindle activity correlates with learning or aging, or whether it is associated with sleep stability. From all experiments on cat sleep physiology, only the classical "dream demonstration" by Jouvet (1979) attempted to bind physiology to behavior and only pharmacological validity (Nestler and Hyman 2010) is satisfied at the moment (Ganes and Andersen 1975; Ganes 1976). The 7-14 Hz frequency range of feline sigma is reasonably close to the definitions used in humans, but this band has a very low lower boundary. The lowest sigma frequencies proposed for humans are between 8 and 9 Hz (De Gennaro et al. 2008; Bódizs et al. 2009) and most researchers (see discussion by Nonclercq et al. (2013)) set the lower boundary even higher, often between 11 and 12 Hz. The latter might be a cautionary measure to avoid contamination of alleged spindle activity by alpha waves, since the two oscillation types overlap in the 8-12 Hz range (Steriade and Llinás 1988). Steriade and Llinás, however, who were particularly vocal against the notion that alpha and sleep spindles are related (Steriade and Llinás 1988) also consistently defined feline sigma in the 7-14 Hz range. A more relevant issue with face validity in cats is the absence of topographic analogies to humans (discussed by van Luijtelaar (1997)), i.e. a slow versus fast spindle dichotomy was not reported.

The so far most promising model in comparative sleep spindle research is the rat. Face validity is met: not only does the alleged frequency band of rat sigma overlap strongly with the definitions used in human studies, for instance 12-15 Hz in Eschenko et al. (2006), but a distinction between slow and fast spindles, with a similar topographic distribution has also been found (Terrier and Gottesmann 1978). Predictive and external validity are supported by spindle occurrence increasing with learning (Eschenko et al. 2006; Mölle et al. 2009) and coinciding with ripples (Siapas and Wilson 1998). The stricter, pharmacological definition of predictive validity (Nestler and Hyman 2010) is also satisfied (van Luijtelaar 1997). Concerning construct

[19] Combining EEG with fMRI the study found thalamic activation for both fast and slow spindles in humans.

validity, the rat has been mostly used to study the cortical contributions to spindle expression (Kandel and Buzsáki 1997; Sitnikova 2010; Peyrache et al. 2011), but even so, a similar thalamic dependence as demonstrated in the cat (Steriade and Llinás 1988) and implied in humans (Schabus et al. 2007) was also revealed in rats (Meeren et al. 2009). The latter confirms the specific role of the RTN (Reticular Thalamic Nucleus) discussed in cat spindles. Among the few shortcomings of the rat model, is that effects of aging have not been sufficiently studied (van Luijtelaar and Bikbaev 2007; Sitnikova et al. 2014a)[20] and the estrous cycle does not seem to affect sleep spindles in this species (Schwierin et al. 1998).

1.4.2 What we know about canine sleep spindles

Prior to the efforts I will describe in this thesis the dog was unlikely to have been considered a promising model for comparative spindle research, from the perspective of the three validities and the literature up until 2017. However, as discussed earlier in the introduction, the dog is a particularly promising model for non-invasive, comparative brain research (Bunford et al. 2017) which stimulated a more intense effort to change this situation.

Virtually no study in which dog spindles were mentioned had attempted to quantify the events, relate them to any form of behavior, age-related changes, pathological changes or signs of sexual dimorphism (Petersen et al. 1964; Pellegrino and Sica 2004; Jeserevics et al. 2007; Pákozdy et al. 2012; Kis et al. 2014). In some of these papers a discussion of sleep spindles was reduced almost entirely to the figure legends (Pákozdy et al. 2012; Kis et al. 2014). One notable exception was a study comparing the brain development of rabbits, cats and dogs (Petersen et al. 1964). Although Petersen et al. did not quantify spindle occurrence, amplitude, duration or frequency either, the time (measured in post-natal weeks) they first became visible in the EEG signal was documented. It was found that dogs display spindles later than cats and rabbits.

Attempts to define canine spindles in terms of frequency vary strongly. Pákozdy et al. (2012) and Pellegrino and Sica (2004) describe spindling events[21] between 5-12 Hz and 6-12 Hz, respectively, which are of much lower frequency than in humans. More similar to humans, Jeserevics et al. (2007) describe spindling events of up to 15 Hz in the dog. Also a figure legend in Kis et al. confirms 12-16 Hz, thus also more similar to human sigma, spindle-like events. In the main text of this paper spindles were merely mentioned as a criterion for identifying sleep stages, but not quantified. It is possible that the apparent contradiction is due to the use of

[20] So far only in animals that where epileptic and/or younger than 9 months.
[21] But actually only Pákozdy literally calls them spindles.

anesthesia by Pákozdy et al. (2012) and Pellegrino and Sica (2004) (see Steriade and Llinás (1988)), but note, that in the original cat studies spindles as low as 7 Hz were dependent on the intrinsic dynamics of the RTN (Steriade et al. 1985; Bazhenov et al. 1999). The most puzzling estimate of canine spindling frequency is found in one of the early editions of a chapter on mammalian sleep (Kryger et al. 2011) and reads 2-5 Hz. The value is corrected in later editions to "11 waves per second". It is convenient to assume that the first edition contained an error. In neither edition is the claim tied to a citation, and frequencies as low as 2-5 Hz should be undistinguishable from delta waves which are universally around 1-4 Hz in mammals (Genzel et al. 2014). The latter is also confirmed for dogs (Kis et al. 2014). However, curiously, this is not an isolated instance of a commonly studied oscillation being reported as displaying a surprisingly low frequency in the dog. Pampiglione (1977) reports in young dogs a pattern that appears under the condition which usually is associated with alpha activity in humans (thus 8-12 Hz). That is, he described a pattern visible over the posterior part of the head, during quiet wakefulness and in particular when the animal's eyes are closed. In dogs younger than 5 months, however, this alpha-like rhythm was only 4-6 Hz slow and merely increased to 6-8 Hz later on. This is much lower than even some alpha definitions used in later dog EEG studies[22] (Wauquier et al. 1988).

1.5 Specific goals and the general procedure

The thesis will seek to tackle three specific goals. Firstly, to develop an automatic detection algorithm for sleep spindles in dogs and evaluate its reliability and external validity by comparing its performance across two data sets and with the literature. Secondly, to evaluate if spindle-like bursts in the dog are analogue to human sleep spindles using the three validities originally outlined in the study of disease models and their more broadly redefined meaning explained above. Last, but not least, if goals one and two are satisfied, we will explore what characterizes spindle expression in dogs based on the findings discussed in this thesis and what this tells us about mammalian sleep evolution in general and canine neuropsychology in particular.

Below I will first describe the methods that apply to both investigations I will report in this thesis, based on Iotchev et al. (2017, 2019). It is not uncommon for research on sleep spindles to reuse data-sets obtained from other investigations (Nonclercq et al. 2013; Ujma et al. 2014; Durka et al. 2015). This is definitely true for the development of detection algorithms

[22] To be precise 7.5-12.5 Hz.

(Nonclercq et al. 2013; Durka et al. 2015), which is a sub-goal of this work as well, but is also an acceptable practice in studies investigating associations between sleep spindles and other variables, see for instance Ujma et al. (2014)[23]. The studies will be discussed in the sequence in which they were published/written, methodological details unique to each study will be discussed in the respective chapter under the heading "subjects, data set and protocol".

Chapter 2: Methods

2.1 Detection algorithm

The following algorithm was used in all of the below described studies. All steps were implemented in Matlab®. Detections were obtained from parts of the EEG marked as non-REM sleep in Kis et al. (2017b) (the individual stages of non-REM sleep are not clearly distinguishable in the dog). The EEG signal (Fz-Cz) was filtered to remove electrical noise and artefacts (maximal frequency of stop band 3 Hz, high pass 5 Hz; low pass 16 Hz, minimal frequency of stop band 35 Hz using a Butterworth filter with less than 0.5 dB ripple in the pass band and 30 dB attenuation), then analyzed with a Fast Fourier Transform (FFT) of 125 ms overlapping, Hanning-tapered 500 ms windows and zero-padded to support a 0.1 Hz resolution. The window length corresponds to the minimum duration of a spindle (Rechtschaffen and Kales 1968) and is in accordance with previous propositions for moving windows (Barlow 1985; Nonclercq et al. 2013) targeting spindle detection. Except for the pre-filtering, all the above steps are adopted from Nonclercq et al. (2013). Because canine EEG recordings are more noisy (Kis et al. 2014, 2017b) and obtained by a single bipolar derivation (Kis et al. 2014), our filter settings excluded more frequencies than proposed in the original description of the method (Nonclercq et al. 2013) in order to ensure a higher signal to noise ratio. In addition, we also repeated each search with one harmonic below and above the target frequency to ensure any effect discovered with that target was not due to random noise or spectrally similar, sharp-wave, epileptoform activity. Three automated criteria were then used to determine if a time-window was occupied by a spindle:

1. The maximum peak power of a given segment was within the target range (Nonclercq et al. 2013) (12-14 Hz (Rechtschaffen and Kales 1968), 9-16 Hz (Bódizs et al. 2009), 5-12 Hz (Pákozdy et al. 2012) respectively).

[23] Although in Ujma et al. (2014) a subset of recordings was made specifically for the study. This is also true for our 2nd investigation.

2. Amplitudes were calculated for each window as the root mean square (rms) of the corresponding signal segment (Nonclercq et al. 2013). The amplitude of a spindle was previously proposed to be one standard deviation from the amplitude of the baseline (Rechtschaffen and Kales 1968; Nonclercq et al. 2013), which was the filtered total of non-REM epochs (Nonclercq et al. 2013). Because the EEG traces displayed high inter-individual variation in amplitude, standard scores (Z) were calculated for each window's rms value relative to the population of rms values for that dog and session. If the standard score for a window was ≥ 1 the event was considered large enough to be part of spindle activity. For the control search based on the harmonics of the target frequencies, the segment was chosen twice as long for the first lower harmonic and half as long for the first higher harmonic.

3. In humans, spindles were previously found to follow a normal distribution in both amplitude and frequency within individuals (Zeitlhofer et al. 1997; Nonclercq et al. 2013). To account for each subject's individual range on these measures, for both amplitude and frequency the true means and standard deviations of an individual dog and session were estimated using a maximum likelihood estimation on the sample of detections obtained with step 1 and 2. This was followed by an altered repetition of the first two criteria: The frequency boundaries of the first criterion were redefined as ± 2 standard deviations from the estimated mean and the same was done for amplitude based on the amplitude's estimated mean and standard deviation (using the standard scores from the 2nd criterion). Due to the latter adjustments the final group of detections could include events with frequencies outside the initial search range, while events outside the adjusted range were discarded as outliers. The events were required to also fulfil the original 2nd criterion with rms standard scores being above 1.

The time-windows whose content passed these three criteria and two consecutive selections, were used to select from a corresponding array of time-points referencing the center of each time-window. To automatically estimate the number of separate spindles, the selected time-points that were less than half a second apart (Nonclercq et al. 2013) were grouped as referring to the same spindle-event. The average intra-spindle frequency was, however, calculated across all time-windows containing spindle-activity. Amplitudes were measured as the relative distance to baseline, using the standard scores calculated for criterion 2. The time-points were also collected by our algorithm and used to visualize the detections in 15-second-long windows

(Figure 6). Detections were also separated into fast and slow spindles (see introduction) using a 13 Hz threshold (slow ≤ 13 Hz, fast ≥ 13 Hz) as applied by Schabus and colleagues (2006).

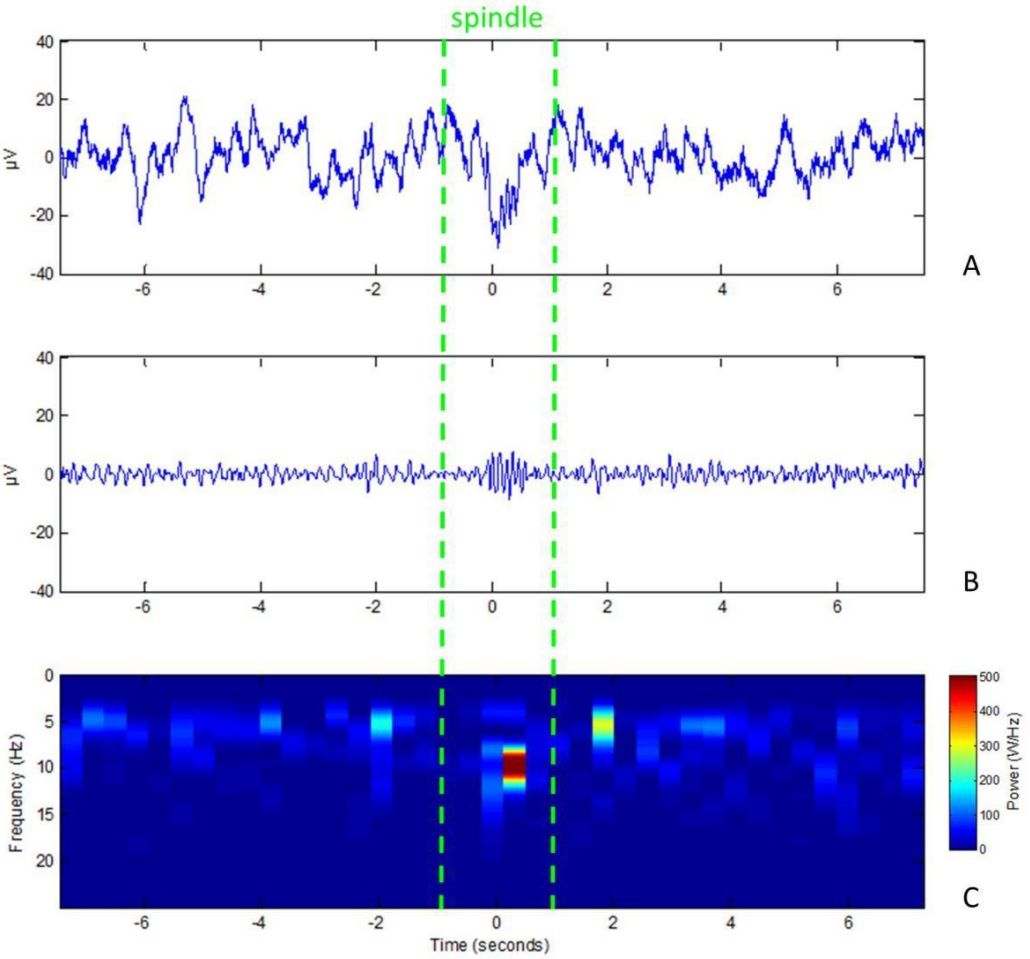


Figure 6. Example of a spindle detected by the algorithm, as it appears in the raw signal (A), in the filtered signal (B), and the change in frequency-power for the same segment (C). Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

2.2 Polysomnographic method

The polysomnographic method used in the studies reported here was originally devised by Kis et al. (2014). Discussing polysomnographic technique in depth is outside the scope of this thesis, but below we will describe the most relevant points for understanding our findings. We still advise to attend to the original method paper (Kis et al. 2014) for additional details where it is of interest to the reader.

Invariantly, three EEG electrodes were placed on the skull midline (referred to later on as Fz, Cz, Pz, i.e. 'frontal', 'central' and 'posterior' midline (z) electrodes, see also Figure 7). The anterior midline electrode (Fz) was active in all dogs, but Cz was active in only 86

animals in our 2nd study (Iotchev et al. 2019) (55.5% of the total sample) and all animals in our third study (Iotchev et al, in prep.). Both Fz and Cz (where applicable) was referred to Pz, placed on the occipital bone at the back of the dog's head. The remaining head electrodes consisted of a ground electrode at the left musculus temporalis and one or two additional electrodes for measuring eye movements (placed on the left and right os zygomioticum). Furthermore electrocardiogram (ECG), respiration and muscle tone was monitored in order to aid sleep stage identification. The impedance of the active electrodes was kept below 20 kΩ. Furthermore, recordings in our database were obtained with one of the following two technical arrangements:

(1) In 17.4% of the total sample in our 2nd study (Iotchev et al. 2019) and 100% of the dogs in our 1st study (Iotchev et al. 2017) the signal was collected, pre-filtered, amplified and digitalized with a sampling rate of 249 Hz/channel using a 30-channel Flat Style SLEEP La Mont Headbox with implemented second order filters (high pass > 0.5 Hz, low pass < 70 Hz), and HBX32-SLP 32 channel pre-amplifier (La Mont Medical Inc., USA).

(2) In 82.6% of the total sample in our 2nd study (Iotchev et al. 2019) the signal was collected, pre-filtered, amplified and digitized with a sampling rate of 1024 Hz/channel using a SAM 25 R style MicroMed Headbox (MicroMed Inc., Houston, TX, USA). The hardware passband was set at 0.5–256 Hz, sampling rate of 512 Hz, anti-aliasing filter with cut-off frequency at 1 kHz, and 12-bit resolution covering a voltage range of ± 2 mV as well as second-order software filters (high pass > 0.016 Hz, low pass < 70 Hz) using System Plus Evolution software (MicroMed Inc, Houston, TX, USA).

To account for the use of different recording set-ups, we had to switch from a discrete time zero-pole-gain representation to a second-order section representation of the Butterworth filter for offline filtering (in the 2nd and 3rd study), while keeping the same attenuation coefficients, pass band and stop band cut-off frequencies, as previously described (Iotchev et al. 2017). Second-order section representations of filters are based on equations that provide transfer-functions less sensitive for individual deviations in how different signal components are amplified or attenuated during sampling with different settings. As an additional control against systematic differences due to recording method, spindle measures were compared between the two groups in the supplementary (supplementary to chapter 4). Prior to filtering, the EEG signal

was divided in sleep-stages using visual inspection, as in Kis et al. (2017b) with the EEG viewing program *Fercio's EEG Plus*.

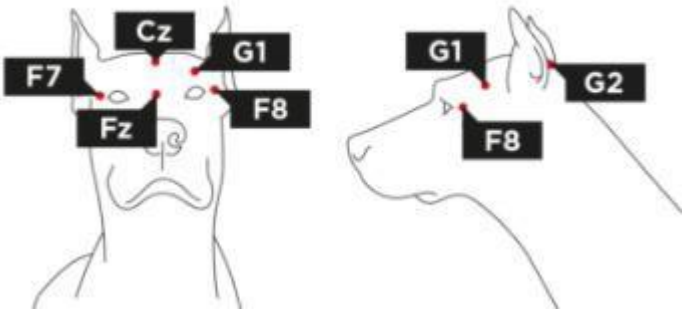


Figure 7. Placement of EEG (Fz, Cz), reference (G2), ground (G1) and eye-movement (F7, F8) electrodes according to the method of Kis et al. (2014). Adopted from Iotchev et al. (2019) originally drawn by Vivien Reicher. Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

Chapter 3: Sleep spindles and post-sleep recall in the dog

3.1 Context and goal of the study

Our first investigation into canine sleep spindles[24] had two goals, Firstly, to clarify the exact frequency range of canine spindles for which the estimates varied widely in the literature (Kryger et al. 2011; Pákozdy et al. 2012; Kis et al. 2014). While this variation can partly be explained by differences in methodology (see Introduction), it should be noted that a universal frequency range for mammalian sleep spindles is usually neither reported nor expected (Kryger et al. 2011), nor is it the case that analogous EEG signatures are always displaying the same frequency across species (see for example Coenen and van Luijtelaar (2003)). Secondly, we wanted to confirm if in dogs the occurrence of sleep spindles, measured below as density (detections/minute), bears the same relationship to recall performance as in humans. That is, if occurrence increases with exposure to new information (Gais et al. 2002; Schmidt et al. 2006; Schabus et al. 2008; Mölle et al. 2009) and correlates with post-sleep improvement on the novel task (Gais et al. 2002; Clemens et al. 2005, 2006; Schmidt et al. 2006; Cox et al. 2012; Lustenberger et al. 2016). Moreover, we wanted to use results concerning the second question towards answering the first. We reasoned that the true canine spindle should display a similar relationship to recall performance as human sleep spindles do. We chose to approach the twofold goal with automatic detection (see Methods) and without post-hoc rejection of atypical

[24] Published originally under the title "EEG Transients in the Sigma Range During non-REM Sleep Predict Learning in Dogs" in *Scientific Reports* (Iotchev et al. 2017).

detections, which we reasoned was the best way to avoid confirmation bias. We also chose a narrow set of hypotheses about the true frequency range of canine spindles, derived from the literature, as to reduce the risk of committing a circular reasoning fallacy. We compared, specifically, three hypotheses. The first hypothesis states that canine spindles are markedly different from human spindles, as the 5-12 Hz estimate obtained by Pákozdy et al. (2012) suggests. The second and third hypotheses were instead based on the assumption of a similar spindle frequency between humans and dogs, comparing the narrow 12-14 Hz definition (for instance used in Nonclercq et al. (2013)) with a broader range, as exemplified by the 9-16 Hz used in Bódizs et al. (2009). The data set for the investigation was taken (with permission) from Kis et al. (2017b) as it allowed us to investigate simultaneously if spindle occurrence increased with exposure to novel information and also if it predicts recall performance (similar to Gais et al. (2002)). Below we include a description of the data and results adopted with minor changes (and permission) from Iotchev et al. (2017).

3.2 Subjects, data set and protocol

15 adult pet dogs, age range: 1-8 years, M ± SD: 3.87 ± 2.17; 7 female; from 7 pure breeds (3 Border Collies, 2 Golden Retrievers, 1 Labrador Retriever, 1 Poodle, 1 Belgian Shepherd, 1 Puli, 1 Miniature Schnauzer) and 3 mixed breeds (3 unknown, 1 mixed Briard and 1 mixed Malinois), participated in the study.

An adaptation was followed by a control (non-learning) and learning session in a counterbalanced order (a total of three conditions). The dogs had three hours' time to sleep on each of these occasions and the EEG data were obtained from these sleeping periods (see Figure 8).

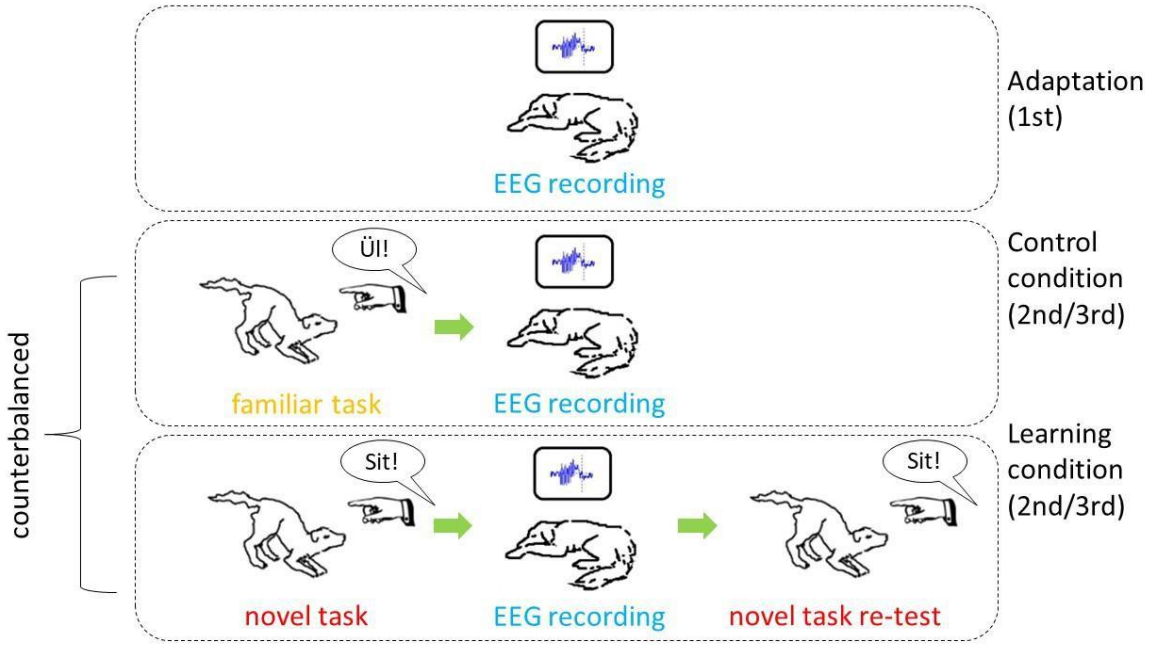


Figure 8. A schematic overview of the structure and sequence of conditions in Kis et al. (2017b) and Iotchev et al. (2017). Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

7 out of 15 dogs (46.7%, group I) started with the control (non-learning) condition first. Dogs of group II received the reversed order. The interval between the two sessions was on average 10.5 ± 2.9 days (mean ± SE, range: 1-38) with no difference between the groups.

In the learning condition dogs were taught to perform two familiar actions (sit and lie down), using unfamiliar commands (English phrases instead of Hungarian, the language in which the dogs were initially trained). The training procedure followed a standardized schedule concluding with an 18-trial baseline test (for details see supplementary in Kis et al. (2017b)). In the non-learning condition the dogs had to execute an identical sequence of "Sit!" and "Lie down!" actions, but this time the experimenter used the familiar commands, accompanied by familiar hand signals. The learning and non-learning tasks were followed by a 3-hour-long polysomnography recording each. In the learning condition, the polysomnography recording was followed by a second 18-trial session in which dogs were tested once more with the previously learned English commands. Performance was measured in each condition as the percentage of correctly followed commands (but see supplementary Table S1 for the absolute number correct responses in each session). The variable 'learning gain' was used to measure recall performance in the learning condition, calculated as the difference of performance scores before and after sleep.

3.3 Statistical analysis

A generalized linear mixed model (GLMM) was used for comparisons involving more than one predictor. The model used a robust estimation and a Satterthwaite approximation for the degrees of freedom, which is recommended for small sample sizes (N = 15, unless otherwise stated). A Gamma distribution assumption was chosen for most readout variables, since negative values cannot be scored for frequency, amplitude or density (Gamma is recommended for distributions with all positive values). One exception was learning gain (one dog performed worse after sleep) and for this variable the default linear assumption was used, after normality was visually confirmed. Significant effects were further analyzed post-hoc, either using the build-in post hoc tests of the GLMM (for categorical variables) or running the model again with a single predictor. Paired-sample t-tests were used for comparisons between conditions. Results were plotted if significant in post-hoc testing (GraphPad Prism).

3.4 Results

For a descriptive exploration of the characteristics of sleep spindles we used the data from the adaptation sessions. Three different criteria for spindles were explored, see Table 1.

search range	detections	detections/ minute	frequency (Hz)	amplitude (z-score)
12-14 Hz (Nonclercq et al. 2013)	8.7 ± 3.02	0.4 ± 0.1	12.9 ± 0.1	1.6 ± 0.1
9-16 Hz (Bódizs et al. 2009)	71.1 ± 18.4	3.1 ± 0.4	9.9 ± 0.3	1.9 ± 0.2
5-12 Hz (Pákozdy et al. 2012)	202.7 ± 51.8	8.6 ± 1.1	6.2 ± 0.1	1.8 ± 0.1

Table 1. Means and standard errors for the absolute number of detections, occurrence per minute non-REM sleep, their average frequency, and amplitude, shown for each target range tested. On average non-REM sleep in the adaptation session lasted 32.1 ± 7.4 minutes (N = 12, 3 dogs did not sleep and were excluded for calculating the average frequency and amplitude). Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

3.4.1 Age, sex, and learning gain

An initial exploration into how learning gain (difference in percentage correct responses after sleep – before sleep in the learning condition) was predicted by sex and age revealed no effect of age (GLMM, $F_{1,12} = 0.075$, $P = 0.789$), but a significant effect of sex (GLMM, $F_{1,12} = 5.591$, $P = 0.036$). Females displayed a higher learning gain (15.6 ± 3.6 versus 4.4 ± 2.2, means ± SE, $t_{12} = 2.636$, $P = 0.022$), see Figure 9B.

Next, the overall predictive strength of detections from each frequency-definition was compared by testing how age, sex and learning gain would predict spindle density in the learning condition. Transients in the 5-12 Hz and 12-14 Hz range showed no relationship to learning or age (see Supplementary). Below we present the results for transients in the 9-16 Hz range (Figure 9).

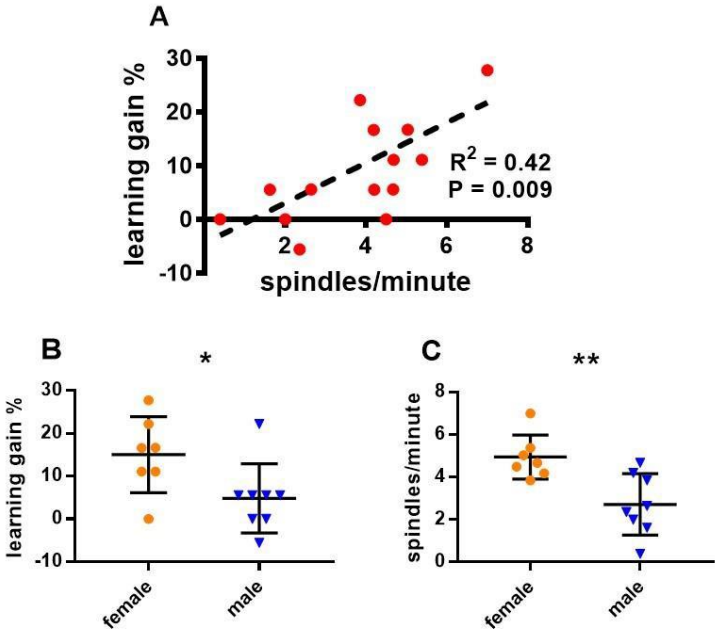


Figure 9. Correlation spindles/minute and learning gain (A). Scatter plots for the difference between male and female dogs in learning gain (B) and spindles/minute (C). Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

We found that spindle density in the learning condition increased with learning gain (GLMM, $F_{1,11} = 8.798$, P = 0.013). This relationship remained significant in post-hoc testing (GLMM, $F_{1,13} = 9.293$, P = 0.009, Figure 9A). Spindle density also increased with age (GLMM, $F_{1,11} = 7.869$, P = 0.017) and was different for the sexes (GLMM, $F_{1,11} = 10.956$, P = 0.007). Females had a higher spindle density than males (4.75 ± 0.2 versus 2.69 ± 0.4, means ± SE, $t_{11} = 4.787$, P = 0.001, Figure 9C), but the effect of age was not significant post-hoc (GLMM, $F_{1,13} = 0.879$, P = 0.366).

The difference in density between conditions was tested using paired-sample t-tests. For detections in the 12-14 Hz range the difference was non-significant ($t_{14} = 1.002$, P = 0.333) and detections in the 5-12 Hz likewise did not display a different density for learning versus control ($t_{14} = 1.139$, P = 0.274). We found a significant increase in density in the learning condition as compared to the non-learning condition for detections in the 9-16 Hz range (3.76 ± 0.43 versus

2.87 ± 0.47, means ± SE, t_{14} = 2.264 P = 0.04, Figure 10A). To account for the distance between sessions, we repeated the density comparison for dogs with less than 10 days between the two sessions (excluded dogs N = 4, ≥ 13 days between sessions). The difference in density remained significant (3.62 ± 0.59 versus 2.23 ± 0.52, means ± SE, t_{10} =3.114 P = 0.01, Figure 10B).

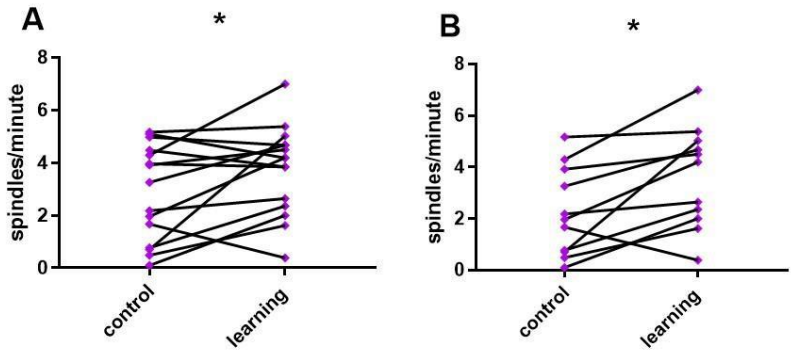


Figure 10. Difference spindles/minute between the control and learning condition, for all dogs (A) and dogs with less than 10 days between experimental sessions (B). Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

To examine if the effect of sex was dependent on an intact reproductive system we used independent samples t-tests in both conditions, excluding neutered animals (N = 10, 4 ♀). While the effect was visible for all frequency definitions, we concentrated on detections in the 9-16 Hz range due to their significant association with learning within and between conditions. Females displayed a significantly higher density of spindles in the control condition (3.5 ± 1 versus 1.2 ± 0.4, means ± SE, t_8 = 2.627, P = 0.03, Figure 11A) as well as the learning condition (5.5 ± 0.5 versus 2.2 ± 0.5, means ± SE, t_8 = 4.259, P = 0.003, Figure 11B). No data was available on the estrous cycle of intact females.

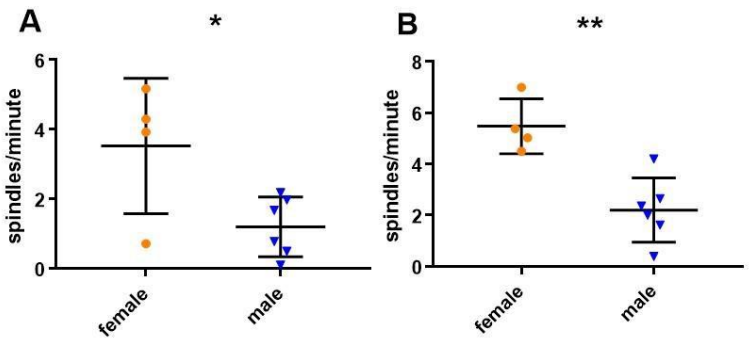


Figure 11. Scatter plots for the difference in spindles/minute between female and male dogs in the control condition (A) and learning condition (B). Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

3.4.2 Slow and fast spindles

The next set of tests aimed to inquire if a distinction between fast and slow spindles, now a standard in the human literature (De Gennaro and Ferrara 2003), would yield meaningful information in the dog. To this end we focused on the 9-16 Hz range which is deemed to encompass both subtypes (Bódizs et al. 2009). Using the 13 Hz mark (see Schabus et al.(2006)), we divided the previously obtained 9-16 Hz transients in slow spindles (<= 13 Hz) and fast spindles (>= 13 Hz). The GLMM for effects of learning gain, age and sex in the learning condition as well as the paired samples t-tests of spindle density between conditions was repeated to establish which variety was more specifically connected to learning. In addition, we averaged the amplitudes, frequencies and densities across both conditions and used a GLMM to see if they could be used to predict age, excluding dogs with no detections (3 dogs had no fast spindles, N = 12 for the fast spindles analysis). Because the frequency and amplitude of slow and fast spindles could vary independently as the literature suggests (De Gennaro and Ferrara 2003; Ujma et al. 2014) we investigated these features for fast and slow spindles separately.

1. Slow spindles: In the learning condition the density of slow spindles was significantly predicted by learning gain (GLMM, $F_{1,11} = 10.634$, $P = 0.008$). This effect was also significant post-hoc (GLMM, $F_{1,13} = 11.661$, $P = 0.005$, Figure 12A). Sex was a

significant predictor (GLMM, $F_{1,11}$ = 5.419, P = 0.04). Females had more spindles/minute than males (4.1 ± 0.3 versus 2.6 ± 0.4, means ± SE, t_{11} = 3.031, P = 0.011, Figure 12B). Density increased with age (GLMM, $F_{1,11}$ = 6.144, P = 0.031), but this effect was not significant post-hoc (GLMM, $F_{1,13}$ = 0.661, P = 0.431). There was a trend for more spindles/minute in the learning condition as compared to the control condition (3.4 ± 0.4 versus 2.6 ± 0.5, means ± SE, t_{14} = 2.135, P = 0.051). This effect was significant upon excluding dogs with more than 10 days waiting time between the EEG sessions (3.2 ± 0.5 versus 2.01 ± 0.5, means ± SE, t_{10} = 2.959, P = 0.014, Figure 12C). Age was not predicted by the mean amplitude (GLMM, $F_{1,11}$ = 0.257, P = 0.622), mean frequency (GLMM, $F_{1,11}$ = 0.268, P = 0.615) or mean density of slow spindles (GLMM, $F_{1,11}$ = 0.003, P = 0.959).

2. Fast spindles: The density of fast spindles was not predicted by learning gain (GLMM, $F_{1,9}$ = 0.001, P = 0.973) or age (GLMM, $F_{1,9}$ = 0.138, P = 0.719) but was significantly predicted by sex (GLMM, $F_{1,9}$ = 11.521, P = 0.008). Females displayed more fast spindles/minute than males (0.8 ± 0.2 versus 0.2 ± 0.1, means ± SE, t_9 = 2.631, P = 0.027, Figure 12D). Condition had no effect on the density of fast spindles (t_{14} = 1.557, P = 0.142). Age was predicted by the mean amplitude of fast spindles (GLMM, $F_{1,8}$ = 27.651, P = 0.001), which decreased with years of age. There was trend for their density to decrease as well (GLMM, $F_{1,8}$ = 3.516, P = 0.098), while frequency did not predict age (GLMM, $F_{1,8}$ = 2.502, P = 0.152). The effect of density was not significant post-hoc (GLMM, $F_{1,10}$ = 0.132, P = 0.724), but mean amplitude remained a significant predictor of age (1.7 ± 0.1 versus 1.6 ± 0.2, means ± SE for dogs older and younger than 6 years (Wallis et al. 2016), amplitude measured as standard deviation above baseline; GLMM, $F_{1,10}$ = 17.454, P = 0.002). See Figure 12 for a summary of these results.

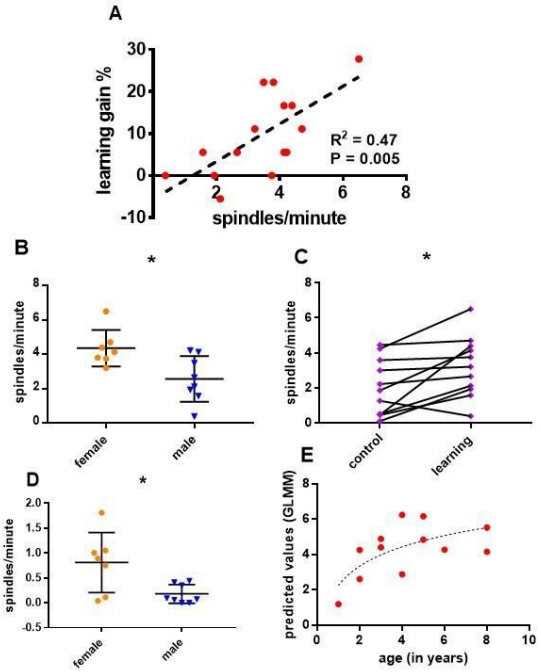


Figure 12. Correlation slow spindles/minute and learning gain (A), scatter plots for the difference between female and male dogs in spindles/minute for slow (B) and fast (D) spindles in the learning condition. Difference of slow spindles/minute between learning and control conditions, excluding dogs with more than 10 days between sessions (C). Association between age (in years) and predicted values for fast spindle density, amplitude and frequency (E). Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

3.5 Discussion

In our first study we confirmed two well-established associations between sleep spindles and learning in a novel species. An increase in spindle occurrence, measured here as density (spindles/minute), was higher when dogs were exposed to novel learning challenges prior sleep, as was observed in humans and rats (Gais et al. 2002; Eschenko et al. 2006; Schmidt et al. 2006; Schabus et al. 2008; Mölle et al. 2009). Also similar to several findings in humans, occurrence measured during a sleep taking place between acquisition and recall predicted recall performance (Gais et al. 2002; Clemens et al. 2005, 2006; Schmidt et al. 2006; Cox et al. 2012; Lustenberger et al. 2016). Importantly, both associations between spindle occurrence and learning were specific to the 9-16 Hz, broad definition of sigma (Bódizs et al. 2009). Our control analyses (see Supplementary) further confirm that the findings are not caused by epileptoform artefacts (tested with higher and lower harmonics), nor alpha activity (as defined by Wauquier et al. (1988)). It is not uncommon for a correlation between spindle occurrence and recall performance to show up in similarly small samples in the human literature (N = 15 in Clemens et al. (2006); N = 19 in Clemens et al. (2005), Seeck-Hirschner et al. (2012) and Cox et al. (2012); N = 20 in Hennies et al. (2016)), nor is it uncommon to observe this effect

without distinguishing fast and slow spindle subtypes (Clemens et al. 2005, 2006; Cox et al. 2012; Seeck-Hirschner et al. 2012). The least usual observation associated with learning, was that upon distinguishing fast and slow spindles, the correlation was only significant for the slow subtype. However, while not observed frequently, an association between recall and slow or frontally detected (and thus predominantly slow) spindles is observed in humans exclusively when verbal material is used to study memory performance (Clemens et al. 2005; Schmidt et al. 2006; Kuula et al. 2019). Our set-up also uses verbal material, yet although we do not investigate if actual verbal learning is taking place, the observation aligns with other work suggesting humanlike processing of verbal information in the dog (Andics et al. 2016). Another interpretation supported by the literature is that the task was simply challenging for the dogs (see Schabus et al. (2008) and Schmidt et al. (2006)).

We also observed a higher occurrence of spindles/minute in female dogs. The effect was stronger in the learning condition and if neutered animals were excluded, it was also independently observed for each the slow and fast spindle subtype. This effect is in agreement with some human data (Gaillard and Blois 1981; Ackermann et al. 2015), but see Ujma et al. (2014) and Bódizs (2017). Importantly, in humans, the occurrence of (fast) spindles changes throughout the menstrual cycle (Driver et al. 1996; Baker et al. 2007; De Zambotti et al. 2015) which could complicate female-male comparisons.

Chapter 4: Effects of age, sex and reproductive status on spindle expression in the dog

4.1 Context and goal of the study

With regard to studying aging, it is common in the human literature to use larger samples (N = 114 in Martin et al. (2012), N = 68 in Guazzelli et al. (1986)) and/or spontaneous sleep data obtained in the absence of experimental manipulation. Our group had access and was contributing to a growing data-base of baseline (pre-experimental) polysomnographic recordings from dogs of varying age, sex and reproductive status which would go on to be included in different EEG investigations after an initial recording of their polysomnographic data. At the time of the study[25] we had 155 dogs ready for analysis. The goal was to explore

[25] Published originally under the title "Age-related differences and sexual dimorphism in canine sleep spindles" in *Scientific Reports* (Iotchev et al. 2019).

how spontaneously expressed spindle density, amplitude and frequency vary with age, sex and reproductive status in the dog.

4.2 Subjects, data set and protocol

155[26] dogs (age range 1-16 years, 7.6 ± 4 (M ± SD); 76 females; 107 neutered and 11 of unknown reproductive status; 96 purebred from 39 different breeds) were taken from our Family Dog Project database consisting of ca. 3 hour long, first-time polysomnographic recordings with no additional experimental manipulations. Dogs that did not sleep during the recording (N = 8 in the full sample, N = 2 in the subsample with an active Cz electrode) were excluded from all analyses, while dogs that slept but did not express spindles (N = 1 in the full sample, none in the subsample with active Cz electrode) were also excluded from analyses of amplitude and frequency. For analyses focusing on fast spindles (≥ 13 Hz) more dogs were excluded from amplitude and frequency comparisons (additional N = 20 in the full sample, 7 in the subsample with an active Cz electrode) due to a higher proportion of dogs displaying no fast spindles. One additional dog was excluded in the amplitude and frequency analyses for slow spindles, as it only showed detections ≥ 13 Hz.

The data was gathered between 2012 and 2019. During this period the recording methods and electrode placements had changed. As a result, Cz was active in only 55.5% of the sample, while 82.6% of the sample was using overall newer settings (1024 Hz sampling rate, see also our discussion of the methods used across studies, above).

4.3 Statistical analysis

Independent samples t-tests were used to inquire if dogs of different sex and reproductive status were of significantly different age (in years). This was done to later exclude the possibility that age-effects are potentially explained by sex or reproductive status. Topographic differences in spindle features between Fz and Cz were tested using paired t-tests on the sub-sample of dogs (N = 84) that had data from both derivations. To test how spindle features (density, amplitude, frequency) might differ across age we used Generalized Linear Models (GLM) with robust model estimation, using age (in years) as a covariate, adding sex and reproductive status as fixed factors, and testing for the interactions sex × age and sex × reproductive status. The models were optimized with backwards elimination, excluding the least significant factors first (starting with interactions and keeping factors that are involved in significant interactions), until reaching the lowest absolute value for the Akaike criterion of model evaluation. The last

[26] 15 dogs had taken part in Iotchev et al. (2017), different recordings of these dogs were used in each study, however.

factor removal was reversed if it resulted in a worse Akaike value and the final model is reported. Prior to testing, the residuals obtained for the initial model were examined for deviations from a normal distribution. If normality assumptions were violated (Kolmogorov-Smirnov test of normality $P < 0.05$) the distribution assumptions were adjusted to Gamma for spindle amplitudes and frequencies (recommended for variables with no possibility for negative values) and Tweedie for spindle density (recommended for variables with the possibility for zero values, but not negative values). Significant interactions between sex and reproductive status were followed by post-hoc tests comparing the effects of neutering for each sex, and sex differences in intact dogs (excluding dogs of unknown reproductive status). All analyses were repeated for the subtypes of slow and fast spindles, separated as previously (Iotchev et al. 2017) using the criterion applied in studies by Schabus and colleagues (2006; 2018) (fast spindles: spindles oscillating in a frequency ≥ 13 Hz, slow spindles: ≤ 13 Hz). We also repeated all analyses for detections in Fz and Cz. The need for outlier control analyses was determined visually (it appeared necessary in two analyses concerning amplitude) upon which outliers were identified based on standard scores (cases with a standard score above or below 2.68[27] for the variable in question were excluded). To account for a possible impact of the recording settings on spindle-detection, we compared the density, amplitude and frequency of spindles on Fz between two groups defined by recording method (see methods), using independent samples t-tests. Detections on Cz were not compared, because all dogs with an active Cz channel used the same recording settings.

In the Supplementary (see Supplementary to chapter 4), to account for possible artefacts caused by breed diversity in the sample, we also introduced a series of control tests, comparing the largest breed-matched sub-samples (≥ 8 dogs) with each other for differences in age, sex, reproductive status and spindle features (density, amplitude, frequency). ANOVA was applied for continuous dependent variables (age, spindle features), whereas breed-cohort differences in the ratios of the sexes or reproductive status were inquired with z-tests. All analyses were performed with SPSS version 22.0.0.0.

4.4 Results

Age-related associations

type of spindle	characteristic	recording site	observation

[27] The 2.68 threshold is derived from the quartile-based rule for detecting outliers, applied to the value likelihoods expected in a normal distribution (see for example http://www.cs.uni.edu/~campbell/stat/normfact.html).

fast	density	Fz	younger < older
fast	frequency	Cz	younger < older
slow	amplitude	Cz	younger < older (only in females)
slow	frequency	Cz	younger < older (only in males)
slow	density	Cz	younger > older
slow	amplitude	Fz	younger > older
Sex-related associations			
fast	density	Cz	females > males
fast	amplitude	Fz	females > males
fast	density	Fz	females > males (only in intact dogs)
fast	frequency	Fz	females > males (only in intact dogs)
slow	frequency	Cz	females > males (only in intact dogs)
Reproductive status-related associations			
fast	density	Fz	intact > neutered (only in females)
fast	frequency	Fz	intact > neutered (only in females)
fast	frequency	Cz	neutered > intact
fast	density	Fz	neutered > intact (only in males)
slow	frequency	Cz	intact > neutered (only in females)

Table 2. Overview of all significant findings in Iotchev et al. (2019). Adopted from Iotchev et al. (2019). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

4.4.1 Results control analyses

No age difference was found between male and female (t_{153} = 1.16, P = 0.248), nor between intact and neutered dogs (t_{142} = 1.548, P = 0.124).

The range, mean and standard deviations for density (spindles/minute), frequency (Hz) and amplitude (SD from baseline) for the total sample (across age, sex, reproductive status and breed) are provided in Table 5.

recording channel	**spindles/minute (M ± SD; range)**	**spindle amplitude (M ± SD; range)**	**spindle frequency (M ± SD; range)**
Fz (N = 147)	3.6 ± 2.1; 0-11.7	1.9 ± 0.5; 1.3-4.2	10.0 ± 1.2; 8-16
fast spindles	0.5 ± 0.8; 0-4.7	2.1 ± 1.8; 1.1-17.3	13.9 ± 0.6; 13.1-16
slow spindles	3.3 ± 2; 0-11.7	1.8 ± 0.5; 1.2-4.2	9.5 ± 0.7; 7.8-11.7
Fz (N = 84)	4.1 ± 2.2; 0.6-11.7	1.8 ± 0.4; 1.4-3.6	10.0 ± 1.3; 8-16
fast spindles	0.6 ± 1; 0-4.7	1.5 ± 1.8; 1.1-12.6	11.2 ± 5.9; 13.2-16
slow spindles	3.7 ± 2; 0-11.7	1.8 ± 0.4; 1.4-3.6	9.5 ± 0.7; 8-11.7
Cz (N = 84)	4.1 ± 2.3; 0.2-9.7	1.9 ± 0.7; 1.4-7.2	10.9 ± 1.8; 7.9-15.6

fast spindles	1.3 ± 1.7; 0-7.9	2.0 ± 1; 1.2-8.5	14.2 ± 0.8; 13-16.6
slow spindles	3.2 ± 2; 0.2-9.3	1.9 ± 0.8; 1.4-7.7	9.9 ± 0.9; 7.7-12.3

Table 3. Means and standard deviations for the density (spindles/minute), amplitude (measured in standard deviations from baseline) and frequency (in Hz) of detections in the sigma range (9-16 Hz) on midline electrodes Fz (frontal) and Cz (central), middle row is for Fz detections from subjects with an active Cz electrode. Adopted from Iotchev et al. (2019). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

4.4.2 Topographic differences

More fast spindles were detected on Cz than Fz (53.6 ± 8.4 versus 20.5 ± 3.9, M ± SE; t_{83} = 4.314, P < 0.001) and a higher rate of spindles/minute (1.3 ± 0.2 versus 0.6 ± 0.1, M ± SE; t_{83} = 3.966, P < 0.001) was also observed. Furthermore, fast spindles on Cz displayed a higher frequency (14.2 ± 0.1 versus 11.8 ± 0.6, M ± SE; t_{76} = 4.182, P < 0.001) and amplitude (2 ± 0.1 versus 1.6 ± 0.2, M ± SE; t_{76} = 2.472, P = 0.016).

No difference was found between Fz and Cz for the absolute occurrence of slow spindles (t_{83} = 1.056, P = 0.294), but density (spindles/minute) was higher on Fz (3.7 ± 0.2 versus 3.2 ± 0.2, M ± SE; t_{83} = 2.21, P = 0.03), slow spindle frequency was higher on Cz (10 ± 0.1 versus 9.5 ± 0.1, M ± SE; t_{82} = 4.568, P < 0.001). The amplitudes of slow spindles did not differ for detections on Fz and Cz (t_{82} = 1.415, P = 0.161).

The likelihood distribution for the frequency-content of all detections (across all dogs), on Fz and Cz are presented in Fig. 13.

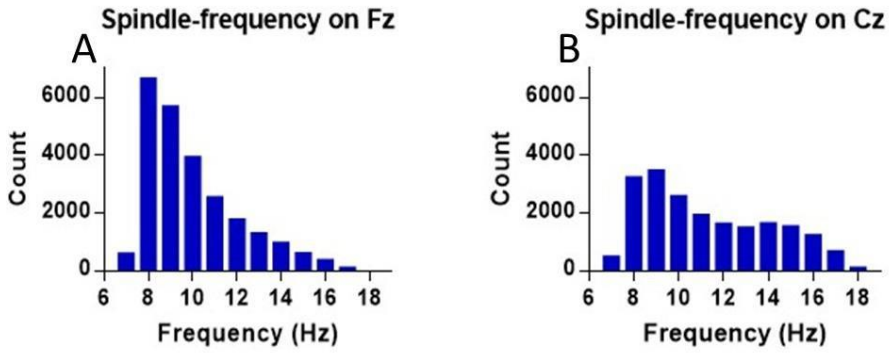


Figure 13. Number of detections for each frequency within the sigma range (9-16 Hz) found on Fz (N = 146, A) and Cz (N = 84, B). Adopted from Iotchev et al. (2019). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

For the GLM analyses we report only the significant findings. For models in which no predictor was significant see supplementary results. Figures summarize all findings relating to a

particular spindling measure (density, amplitude, and frequency) across spindle subtypes and electrodes.

4.4.3 Effects of age, sex and reproductive status on spindle density

For the subset of fast spindles the final model predicting spindle density on Fz included the factors age, sex, reproductive status and the interaction sex × reproductive status. Fast spindle density increased with age (GLM, Wald Chi-Square = 8.107, P = 0.004) and there was an interaction between sex × reproductive status (GLM, Wald Chi-Square = 8.351, P = 0.004). Neutered males displayed a higher fast spindle density than intact males (0.5 ± 0.1 versus 0.2 ± 0.1, M ± SE; GLM, Wald Chi-Square = 5.793, P = 0.016), an opposite trend for more spindles/minute was observed in intact compared to neutered females (0.9 ± 0.3 versus 0.5 ± 0.1, M ± SE; GLM, Wald Chi-Square = 3.127, P = 0.077). Among intact dogs, females displayed a higher density of fast spindles (0.9 ± 0.3 versus 0.2 ± 0.1, M ± SE; GLM, Wald Chi-Square = 12.827, P < 0.001), but there was no difference between male and female dogs among neutered animals (GLM, Wald Chi-Square = 0.061, P = 0.805).

For fast spindle density on Cz, the final model included the predictors sex, reproductive status and age, as well as the interactions sex × reproductive status and sex × age. However, among them only sex was significant (GLM, Wald Chi-Square = 5.588, P = 0.018), females displayed more fast spindles/minute than males (1.5 ± 0.3 versus 1.1 ± 0.2, M ± SE).

The final model for predicting slow spindle density on Cz included only age as a predictor. Slow spindle density declined with age (GLM, Wald Chi-Square = 7.4, P = 0.007). The results for density are summarized in Figure 14.

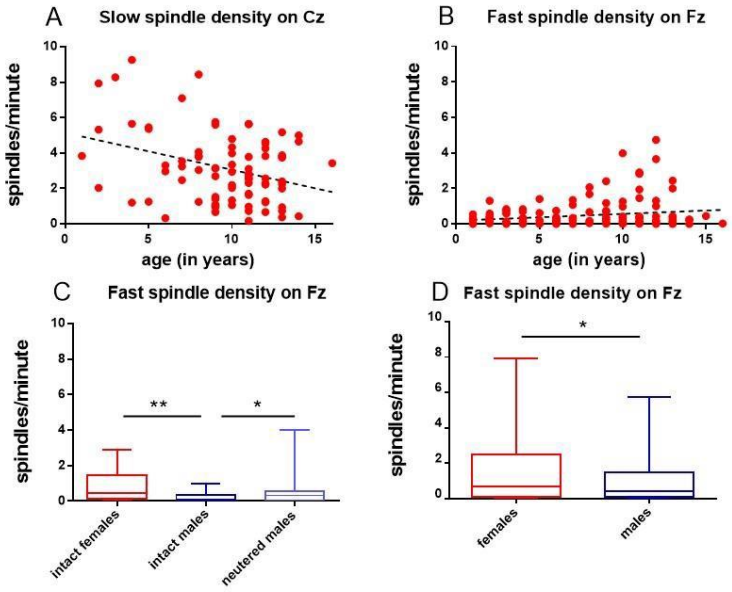


Figure 14. Spindle density as a function of age for slow spindles (≤ 13 Hz) on Cz (N = 84) (A), and fast spindles (≥ 13 Hz) on Fz (N = 147) (B). Boxplot graphs (minimum to maximum and interquartile distance) for fast (≥ 13 Hz) spindle density on Fz, intact females versus intact and neutered males (N = 79) (C); for fast (≥ 13 Hz) spindle density on Cz, female versus male dogs (N = 84) (D). Adopted from Iotchev et al. (2019). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

4.4.4 Effects of age, sex and reproductive status on spindle amplitude

The final model predicting the amplitude of fast (≥ 13 Hz) spindles on Fz included only sex as a predictor (GLM, Wald Chi-Square = 5.724, P < 0.017). Fast spindle amplitude was higher in females compared to males (2.5 ± 0.3 versus 1.8 ± 0.1, M ± SE). The effect remained significant if outliers were removed (GLM, Wald Chi-Square = 5.034, P = 0.025).

The final model predicting the amplitude of slow (≤ 13 Hz) spindles on Fz included the factors age, sex and the interaction sex × age. Slow spindle amplitude declined with age (GLM, Wald Chi-Square = 4.169, P = 0.041).

The final model predicting the amplitude of slow (≤ 13 Hz) spindles on Cz included the factors age, sex, and the interaction sex × age. The interaction sex × age was significant (GLM, Wald Chi-Square = 5.685, P = 0.017). Slow spindle amplitudes on Cz were significantly rising with age in females (GLM, Wald Chi-Square = 7.006, P = 0.008), but not in males (GLM, Wald Chi-Square = 0.109, P = 0.741). Excluding outliers, the age-related increase in amplitude for females remained significant (GLM, Wald Chi-Square = 6.818, P = 0.009). See Figure 15 for a summary of these results.

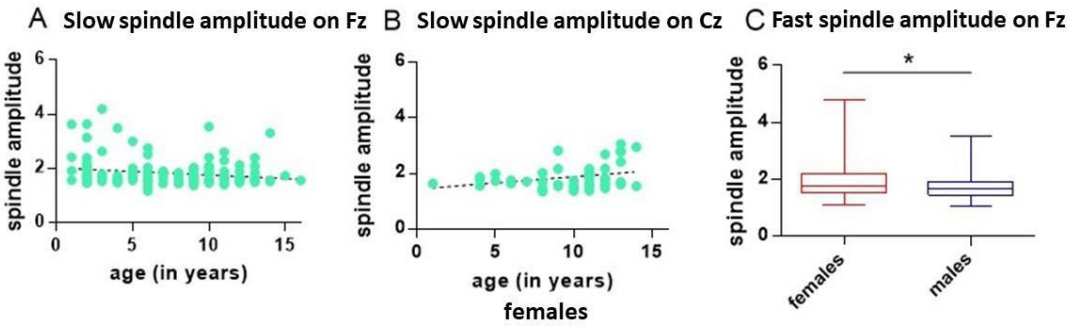


Figure 15. Slow spindle amplitude (≤ 13 Hz) on Fz (N = 145) as a function of age (A). Slow spindle amplitude, in females and on Cz, excluding one outlier (N = 45) (B). Boxplot graphs (minimum to maximum and interquartile distance) for fast (≥ 13 Hz) spindle amplitude, females versus males, for detections on Fz, excluding two outliers (N = 125) (C). Adopted from Iotchev et al. (2019). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

4.4.5 Effects of age, sex and reproductive status on spindle frequency

For fast (≥ 13 Hz) spindles on Fz the final model predicting frequency included the factors sex, reproductive status and the interaction sex × reproductive status. The interaction sex × reproductive status was significant (GLM, Wald Chi-Square = 4.014, P = 0.045). Intact females displayed higher fast spindle frequencies than neutered females (14.3 ± 0.1 versus 13.9 ± 0.1, M ± SE; GLM, Wald Chi-Square = 5.767, P = 0.016), but there was no difference between intact and neutered males (GLM, Wald Chi-Square = 0.385, P = 0.535). Among intact animals females displayed higher fast spindle frequencies than males (14.3 ± 0.1 versus 13.8 ± 0.2, M ± SE; GLM, Wald Chi-Square = 6.366, P = 0.012), but no difference was found between males and females in neutered animals (GLM, Wald Chi-Square = 0.098, P = 0.754).

For fast (≥ 13 Hz) spindle frequency on Cz the final model included the factors reproductive status and age. Fast spindle frequency was found to rise with age (GLM, Wald Chi-Square = 5.666, P = 0.017). Neutered animals displayed higher frequencies than intact animals (14.3 ± 0.1 versus 13.9 ± 0.1, M ± SE; GLM, Wald Chi-Square = 5.343, P = 0.021).

For slow (≤ 13 Hz) spindle frequency on Cz the final model included the predictors age, sex, reproductive status and the interactions sex × age, sex × reproductive status. Slow spindle frequency was predicted by the interaction sex × age (GLM, Wald Chi-Square = 6.023, P = 0.014) and sex × reproductive status (GLM, Wald Chi-Square = 4.406, P = 0.036). Slow spindle frequency on Cz did not change with age for females (GLM, Wald Chi-Square = 0.99, P = 0.32), but was rising in males (GLM, Wald Chi-Square = 7.262, P = 0.007). Among female dogs there was a trend for intact animals to display higher frequencies (10.5 ± 0.2 versus 10 ±

0.2, M ± SE; GLM, Wald Chi-Square = 3.113, P = 0.078) and no difference was found for intact versus neutered animals among males (GLM, Wald Chi-Square = 1.243, P = 0.265). Among intact animals females displayed higher frequencies than males (10.5 ± 0.2 versus 9.6 ± 0.3, M ± SE; GLM, Wald Chi-Square = 5.901, P = 0.015), no sex difference was observed in neutered dogs, however (GLM, Wald Chi-Square = 0.019, P = 0.89). These results are summarized in Figure 16.

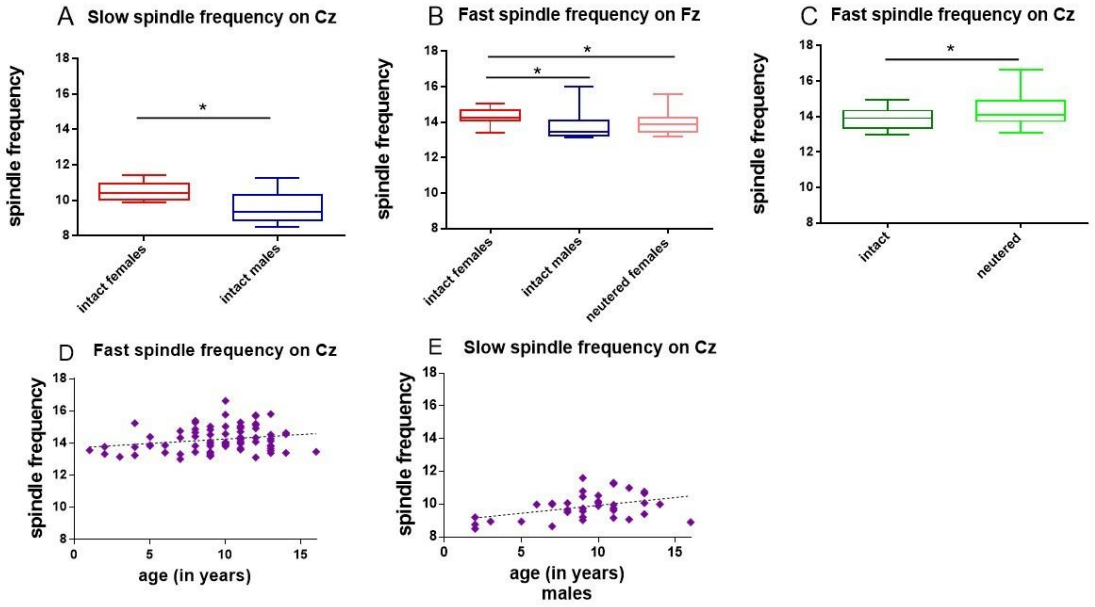


Figure 16. Spindle frequency among intact female and male dogs (N = 15) for slow (≤ 13 Hz) spindles on Cz (A); comparing intact females versus intact males and neutered females for fast spindles on Fz (≥ 13 Hz) (N = 80) (B); comparing all neutered against all intact animals, for fast spindles on Cz (N = 77) (C) boxplot graphs (minimum to maximum and interquartile distance). Fast spindle frequency for all dogs on Cz (N = 77) (D) and frequency of slow spindles in male dogs on Cz (N = 38) as a function of age (E). Adopted from Iotchev et al. (2019). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

4.5 Discussion

The present study not only strengthened (and expanded upon) some observations initially made in Iotchev et al. (2017), but also provided additional arguments for a distinction between fast and slow spindles, which were not confirmed in the cat[28] (Steriade and Llinás 1988), but which, based on what can be observed in humans (Gibbs and Gibbs 1961) and rats (Terrier and Gottesmann 1978), could very well be universal. The first argument is derived from a similar topography, with fast spindles being more abundant when recorded over the more posterior

[28] It also does not seem the case that work in cats aimed to distinguish fast and slow spindles, probably because Steriade and Llinás (1998) found the entire frequency range to rely on the same mechanisms.

derivation, Cz. The other argument derives somewhat more indirectly from our sex and reproductive status related effects.

The previously observed higher occurrence of spindles in female dogs (Iotchev et al. 2017) was replicated here specifically for the fast subtype and in intact animals. More generally the density and frequency of fast spindles was higher for intact females compared to intact males (fast spindle density and frequency on Fz) and neutered females (fast spindle frequency on Fz). Note, that both fast spindle density (Driver et al. 1996; Baker et al. 2007; De Zambotti et al. 2015) and frequency (Bódizs 2017) appear to be under the control of the female steroid progesterone, as both fast spindle activity and progesterone peak in the luteal phase (Baker and Driver 2007). This suggests a similar pharmacological profile for fast spindles in humans and dogs. A hormonal regulation of fast spindle occurrence is also supported by our observation (supplementary) that fast spindle density varies more strongly in the intact female than the intact male group, a variation that could be caused by an intact menstrual cycle. Owner-applied neutering is a readily available proxy for pharmacological intervention in a model animal that is otherwise in an owner's care and protection, hence not likely available for direct pharmacological manipulation. In sum, the indirect arguments for the enhancing effects of progesterone and the topography of fast (and slow) spindles, together provide strong support for the notion that a distinction between fast and slow spindles applies in dogs.

Another finding related to sex was that of larger spindle amplitudes in females as well as an interaction between sex and age on spindle amplitude development. Both align with recent invasive measurements in volunteering epilepsy patients (Bódizs 2017).

Our age-related findings in the dog, based on above arguments for a valid distinction, were reported exclusively per subtype (fast or slow spindles might change differently in healthy and pathological aging, see Rauchs et al. (2008)), whereas results concerning all spindles as a whole were placed in the Supplementary. The amplitude of slow spindles was lower in older dogs on Fz, which resembles several observations in humans. Firstly, age-related amplitude decline is generally a common finding for human spindles (Guazzelli et al. 1986; Landolt and Borbély 2001; Martin et al. 2012; Latreille et al. 2015), but is also more pronounced over anterior derivations in man (Landolt and Borbély 2001; Martin et al. 2012), with at least one study also suggesting that a frontal decrease in spindle amplitude is specific to the slow subtype (Landolt

and Borbély 2001)[29]. Humanlike decrease in occurrence (Smirne et al. 1977; Guazzelli et al. 1986; Crowley et al. 2002; Martin et al. 2012; Latreille et al. 2015; Gorgoni et al. 2016) was also observed, but in the dog seems restricted to the slow type as well, and to detections on Cz. In humans, decline in spindle occurrence seems to be predominantly anterior like amplitude decline (Martin et al. 2012). Authors who observed occurrence decline specifically over posterior derivations in humans were looking at demented subjects and fast spindles (Gorgoni et al. 2016). A previously suggested smaller fast spindle amplitude in older dogs (Iotchev et al. 2017) was not confirmed in our larger sample and is therefore dismissed here as the artefact of an underpowered and preliminary investigation.

An unusual preliminary finding to be confirmed here, is the increase in frontal spindle occurrence with age, albeit that it was more pronounced in slow spindles in Iotchev et al. (2017) and specific to fast spindles in the present work. In our previous study, however, the effect was measured in the learning condition, in which slow spindle occurrence generally increased relative to a control condition, whereas here we compared the expression of spontaneous spindle activity across dogs of different ages. The increase in fast spindle occurrence is puzzling, because in humans such a development is observed from childhood to adolescence (Hahn et al. 2018), whereas in pathological aging fast spindle activity is particularly reduced (Rauchs et al. 2008; Gorgoni et al. 2016). Since mechanistic research in non-invasive model animals is not possible, we can only speculate on the reason for this uniquely canine phenomenon. One possibility is that the increase in spindles with faster frequencies reflects a change in inhibitory strength in the frontal cortex, since spindling frequency is lower when inhibition is stronger (see explanation in Steriade and Llinás (1988)) and this would also align with the lower amplitudes observed on Fz, since spindle amplitude increases with feed-forward cortical inhibition (Sitnikova 2010). This would also explain why only fast spindle density increases with age, while for the full sigma range the density was not significantly different between dogs of different ages. However, it is also unlikely that the adolescent increase and dementia-associated decrease of specifically human fast spindles (Rauchs et al. 2008; Gorgoni et al. 2016; Hahn et al. 2018) is the result of altered inhibition, thus alternative scenarios should be considered. Another possibility is that not all neural mechanisms age relative to the animal's life-span. This second possibility is challenged on first sight by the human-like prognosis of

[29] Landolt and Borbély (2001) do not directly discuss slow spindles and amplitude, however, instead they report reduced power (which is however dervied by squaring amplitude) for the frequency range in which alpha and (slow) spindles overlap.

slow spindle changes in the dog, but slow and fast spindles might be shaped by different mechanisms at least on the level of the cortex (Ayoub et al. 2013).

Finally, age-related increases in human spindling frequency are ,in comparison, reported seldomly (Principe and Smith 1982; Crowley et al. 2002; Ktonas et al. 2007) and are not always confirmed (Feinberg et al. 1967), but have been argued crucial in predicting the onset of dementia (Ktonas et al. 2007). In older dogs, we found higher spindle frequencies over Cz. This was observed for all dogs concerning fast spindles, and for male dogs concerning the slow type. The reason for age-related changes in frequency can also be traced back to at least two alternative scenarios. On one hand the already discussed dependency between overall inhibition strength in the brain and spindle frequency (Steriade and Llinás 1988), but by analogy we can alternatively suppose, that increased frequency can be an adaptation to age-related cell-loss, as observed in the dopaminergic system (Subramaniam et al. 2014)[30]. Again, in a non-invasive animal-model we cannot distinguish these scenarios.

Chapter 5: Unpublished analyses and results

5.1 Background

The following chapter exclusively deals with analyses that were and will be left out of our published work, for reasons of convention, because they were not based on hypotheses, or because they were conducted on the basis of already published studies.

5.2 Bayesian retests as a control

Rationale

Although it is a common practice to demonstrate associations between spindle occurrence and recall performance in small samples (Clemens et al. 2005, 2006; Cox et al. 2012; Seeck-Hirschner et al. 2012; Hennies et al. 2016) as we did in Iotchev et al. (2017), it is a potential concern that the likelihood of false positive significance is also more likely with fewer subjects. Particularly troubling in this regard was the negative finding of Ackermann et al. (2015) due to their large sample, although spindle-learning associations are otherwise a frequent observation in the human literature. One potential advantage of Bayesian statistics is their relative independence of sample size. For instance, because evidence for the null and alternative hypotheses are both being quantified and subsequently compared, we cannot obtain

[30] In this study actually demonstrated for Parkinson disease, which however has been shown in rat models to resemble accelerated brain aging (Khojah et al. 2016).

significance by means of sample size alone, as is the case with classical statistics[31]. Although this common example argument is made for too large samples, I will below use Bayesian testing to specifically double check the findings in our smallest sample (Iotchev et al. 2017), because smaller samples are generally trusted less and because there is literature on Bayesian inference being also better adjusted for smaller samples than commonly used methods (see for example Lee and Song (2004) or Bakken and Bond (2013)).

I retested significant post-hoc findings from Iotchev et al. (2017) using one-sided Bayesian versions of the t-test and Pearson correlation as control/additional post-hoc tests to the ones reported above. One-sided testing is preferred in the Bayesian approach as it provides for better informed priors, moreover the literature provides specific expectations for the direction of spindle-associated effects, but we will also include the results for two-sided testing for a broader overview.

Bayesian testing is not associated with P-values, instead, to assign discrete decision on the results we will use the system proposed by Jeffries (1961) to interpret the BF_{10} (Bayesian factor) which quantifies how much more likely the data is given H1 (alternative hypothesis) over H0 (null hypothesis). According to him a BF_{10} of 1 is considered no evidence; 1-3 is anecdotal evidence; 3-10 is substantial evidence; 10-30 is strong evidence, 30-100 is very strong evidence and >100 is decisive evidence for H1. All analyses are done with JASP, a freeware offered by the University of Amsterdam. Results are reported in the same order as in Iotchev et al. (2017).

Results

9-16 Hz (all spindles):

There was substantial evidence that females scored higher in learning gain than males (BF_{10} = 4.13) and anecdotal evidence that the difference between males and females is larger than zero in either direction (BF_{10} = 2.155).

There was strong evidence that spindle density (spindles/minute) and learning gain were positively correlated (R = .647, BF_{10} = 14.059) and substantial evidence that they were correlated in any direction (BF_{10} = 7.081).

[31] The argument is paraphrased from https://wiki.uva.nl/methodologiewinkel/index.php/Bayesian_analyses.

There was strong evidence that females displayed higher spindle density (spindles/minute) than males (BF_{10} = 17.48) and substantial evidence that they were different in either direction (BF_{10} = 8.817).

There was substantial evidence that spindle density (spindles/minute) was higher in the learning than in the control condition (BF_{10} = 3.562) and anecdotal evidence that the conditions differed in either direction (BF_{10} = 1.83). Excluding animals with 10 or more days between sessions the evidence for a higher spindle density in the learning condition was strong (BF_{10} = 11.589) and for a difference in either direction was substantial (BF_{10} = 5.845).

9-13 Hz (slow spindles):

There was strong evidence that spindle density (spindles/minute) and learning gain were positively correlated for slow spindles (R = .688, BF_{10} = 24.734). The evidence remained strong for correlation in either direction (BF_{10} = 12.416).

There was substantial evidence that spindle density (spindles/minute) was higher for females than males concerning slow spindles (BF_{10} = 8.277). The evidence for a difference in either direction was substantial (BF_{10} = 4.221).

There was anecdotal evidence that spindle density (spindles/minute) was higher for slow spindles in the learning condition, compared to the control condition (BF_{10} = 2.932). The evidence was also anecdotal for a difference in either direction (BF_{10} = 1.516). However, excluding animals with 10 or more days between sessions the evidence was substantial for both versions of the test (one-sided BF_{10} = 8.978, two-sided BF_{10} = 4.541).

13-16 Hz (fast spindles):

There was substantial evidence that spindle density (spindles/minute) was higher for females than males concerning fast spindles (BF_{10} = 7.73). The evidence remained substantial if testing for a difference in either direction (BF_{10} = 3.948).

There was substantial evidence that age and fast spindle amplitude did not correlate negatively (R = .144, BF_{10} = 0.226) and anecdotal evidence for no correlation at all (BF_{10} = 0.359).

Discussion

In hindsight we see some of the interpretations discussed above confirmed by the Bayesian retest control analyses. For instance, the decrease of fast spindle amplitudes with age, found in Iotchev et al. (2017), which was not replicated in our bigger sample, but instead we had found

a decrease specific for slow spindle amplitudes (Iotchev et al. 2019) was hypothesized to be the artefact of a small sample. Indeed this effect also remained poorly supported by the Bayesian post-hoc test, which instead suggests substantial evidence against the claim.

Although the Bayesian tests do not relieve the need for replication, the support for a positive correlation between spindle density and recall performance (called 'learning gain' in Iotchev et al. (2017)) varied between substantial and strong (depending on the formulation of the alternative hypothesis) and was throughout strong when looking at slow spindles specifically. This finding squares with the observation that such an association has been replicated successfully with small samples on several occasions in humans (Clemens et al. 2005, 2006; Cox et al. 2012; Seeck-Hirschner et al. 2012; Hennies et al. 2016), thus suggesting that samples of $N \leq 20$ are not underpowered for this type of investigation.

5.2 Unforeseen observations

Rationale

The reason for leaving out the following findings from our published work, was the lack of hypothesis-support behind these purely explorative analyses. That is, no hypothesis for, nor against the observed relationships is known from the literature on sleep spindles. As yet, we will discuss these findings in the thesis as they might potentially carry relevant information about the detection method (see general discussion at the end of the thesis). The observations were made in the largest data set we had analyzed, reported in Iotchev et al. (2019).

Results

Spindle density (for the entire 9-16 Hz range) was negatively correlated with spindle amplitude on Fz ($R = -.443$, $P < 0.001$) and Cz ($R = -.444$, $P < 0.001$). While density and frequency were positively correlated on Cz ($R = .252$, $P = 0.021$).

For fast spindles (13-16 Hz) density and frequency were positively correlated on Fz ($R = .639$, $P < 0.001$) and Cz ($R = .717$, $P < 0.001$).

For slow spindles (9-13 Hz) density and amplitude were negatively correlated on Fz ($R = -.409$, $P < 0.001$) and Cz ($R = -.343$, $P = 0.001$). Density and frequency were negatively correlated on Cz ($R = -.266$, $P = 0.014$) and so were frequency and amplitude ($R = -.28$, $P = 0.01$).

Discussion

Negative correlations between spindle measures, as most prominently observed here for slow spindles, are not expected based on the literature and therefore cannot be interpreted within the known theoretical framework. The only exception is the negative correlation of slow spindle amplitude and density with frequency, suggesting that similar to what has been observed in the dopaminergic system (Subramaniam et al. 2014), frequency might increase as a compensation for loss of function. However, this possibility was already introduced to explain other results which were obtained through the test of specific hypotheses. In addition negative correlations between frequency and amplitude are generally expected due to the power-law, stating that in EEG frequency and amplitude of the measured signal are always negatively correlated (Buzsáki et al. 2012). That the thalamo-cortical circuits involved in spindle generation could potentially also adjust density and amplitude to each other is not evident from the known mechanisms behind spindle generation (Steriade and Llinás 1988), but could also be an indirect consequence of the power-law.

Chapter 6: General discussion

6.1 Discussion structure

The general discussion of my findings will be organized as follows. I will first discuss the measuring instrument used in above investigations and the evidence that has emerged for its strengths and weaknesses. I will then discuss the dog as a model in comparative sleep spindle research using the modified three validities of disease models (modified for application to model animals in general). Finally, I will discuss how the present work adds to our knowledge of brain evolution in general and sleep physiology and sleep spindles in particular.

6.2 Strengths and weaknesses of the detection algorithm

My automatic detection algorithm was first introduced in Iotchev et al. (2017) and is closely modeled on criteria proposed by Nonclercq et al. (2013). A detailed description can be found in Chapter 2 of this thesis.

The reliability of the instrument is empirically supported by the replication of previous findings: higher fast spindle density in female dogs (Iotchev et al. 2017, 2019)[32] and also higher frontal spindle density in older dogs[33]. Its external validity, in turn, is supported by analogies

[32] Although the samples partly overlap in terms of subjects (and that to a small degree), different recordings are used from the same dogs in Iotchev et al. (2017) and Iotchev et al. (2019).
[33] From the same two studies, but slow spindles in Iotchev et al. (2017) and fast spindles in Iotchev et al. (2019).

between human spindles and detections in the dog, suggesting that the instrument is indeed measuring sleep spindles[34]. The latter will be discussed in more detail when we turn to evaluating dogs as model animals in comparative sleep spindle research.

Potential weaknesses of the instrument are derived from the same features that add to its strength. The normal-modeling procedure, proposed by and adopted from Nonclercq et al. (2013), on one hand takes into account inter-individual variation in spindling frequency and amplitude reported in the human literature (De Gennaro et al. 2008; Bódizs et al. 2009) and also potentially helps exclude alpha activity, which overlaps in the 8-12 Hz band with sigma but should in theory not follow the same distribution parameters as it is generated by different mechanisms (Steriade and Llinás 1988). However, because the number of detections vary strongly in dogs and can be at times very low compared to humans, it is possible that the estimates for the normal distribution underlying amplitude and frequency of canine spindles are biased in dogs with an overall low occurrence rate of spindling events. The latter, we suspect, might also be an explanation for the observation of a negative correlation between spindle density and amplitude (see Chapter 5), i.e. a mathematical artefact caused by an upwards biased estimation of amplitude when density is low.

More work will be needed to estimate the exact error rate of this instrument. Generally, all detection instruments, according to signal detection theory, (Green and Swets 1966) should display a proportion of misses and false positives. Although I am aware of the type of error that can result from this method, one particularly important reason to apply normal-modeling is that it is a better alternative for excluding non-spindle activity, like alpha, which overlaps in the defining frequency parameters with real sleep spindles. Other authors have avoided alpha contamination by either moving up the lower boundary of sigma, setting it at times as high as 12 Hz (Uchida et al. 1994; Nobili et al. 1999; Nonclercq et al. 2013; Genzel et al. 2014) or by excluding alpha frequencies post-hoc (Schimicek et al. 1994). The problem with these approaches is that physiological experiments in the cat had shown that RTN-dependent sigma-like frequencies can display a lower boundary as low as 7 Hz (Steriade et al. 1985, 1987) and there is work in humans, too, suggesting a lower boundary for sigma, at least as low as 8 or 9 Hz (De Gennaro et al. 2008; Bódizs et al. 2009). Considering some arguments in the

[34] This employs the additional assumption that real sleep spindles display similar relationships to other variables across species.

introduction, suggesting that dogs might display an overall lower sigma frequency[35], we decided that the advantage of the here used method in not excluding lower frequency spindles outweighs the potential errors associated with normal-modeling[36].

Moreover, the here reported detection algorithm is also the first attempt to produce results in the dog, after some other attempts were less successful (Kis and Bódizs, personal communication). The success of the instrument, specifically in working with canine EEG signals, is likely due to the use of a relative measure of amplitude (expressed in standard deviations from the overall non-REM signal). Since different dog breeds possess skulls of different shape and thickness we could not have relied on absolute measures of amplitude, as applied, for instance, in Clemens et al. (2005).

6.3 Applying the three validities of disease modeling to comparative spindle research

In the introduction I discussed at length how the narrowly defined face, predictive and construct validities, commonly applied in the evaluation of disease modeling (Overall 2000; Coenen and van Luijtelaar 2003; Vervliet and Raes 2013) can be applied more broadly. We proposed, to this end, to use more abstract definitions of the validities, for instance, instead of narrowly defining predictive validity as the prognosis of pharmacological intervention (compared between model and modelled disease) to more broadly include all predictive relationships between the modelled phenomenon and variables of interest (see a similarly broad definition in van der Staay et al. (2009)). Below we will discuss what the results presented in this thesis tell us about dogs as model animals in comparative sleep spindle research, discussing each validity in the same sequence as presented in the introduction:

6.3.1 Face validity

Face validity refers here to all similarities between model and modeled phenomenon that become apparent at first glance, that is, in the absence of investigating mechanisms or predictive relationships. When comparing representative spindles detected by my algorithm in the dog (Figures 6 and S2) with examples from human EEG (Figure 3) we see a strong resemblance in their appearance. However, because we did not post-hoc exclude detected spindles based on their subjective appearance (our goal, in Iotchev et al. (2017), was to avoid confirmation bias by comparing the performance of the detection algorithm for different target frequencies without human intervention) this is not my strongest argument for the fulfillment

[35] Chief among these arguments are the observations in the more closely related cat (compared to humans and rats), since dog-specific observations were probably not reliable (see ongoing discussion and introduction).
[36] Nonclercq et al. (2013) potentially face the same problem, but their instrument was succesfully valdiated by comparison with expert scorers, which is still a valued validation method (O'Reilly and Nielsen 2015).

of the face validity criteria. The strongest argument comes from the localization differences between fast and slow spindles in the dog, observed in Iotchev et al. (2019). The higher occurrence of fast spindles over the central and posterior parts of the cortex, and of slow spindles over the frontal/anterior lobes, resembles observations from both humans (Gibbs and Gibbs 1961) and rats (Terrier and Gottesmann 1978). As in rats, slow spindles are more numerous overall (Terrier and Gottesmann 1978). Topography is an important feature for evaluating face validity, because it distinguishes otherwise similar oscillations, like sleep spindles and alpha (Steriade and Llinás 1988). Another supportive argument is that the detections were obtained under similar conditions as in humans, which concerns both the search criteria (Nonclercq et al. 2013) and the restriction of the search to non-REM sleep, as is also often done in human studies (Cox et al. 2012; Seeck-Hirschner et al. 2012; Kuula et al. 2019)[37].

6.3.2 Predictive/External validity

Predictive validity is defined here as the sum of all predictive relationships that are similar between the model and modeled phenomenon, a broad definition, found also in van der Staay et al. (2009) and overlapping with his definition of external validity. In the two studies I presented here, I observed predictive relationships that resemble what is known from humans and/or rats. I will categorize these observations into three categories: literal similarities, indirect similarities and partial similarities that differ in one aspect or another from humans, but otherwise follow similar trends.

Similarities that most directly resemble findings in the human literature include the age-related decline in amplitude (Guazzelli et al. 1986; Landolt and Borbély 2001; Martin et al. 2012; Latreille et al. 2015), which in humans, too, is more expressed over frontal derivations (Landolt and Borbély 2001; Martin et al. 2012) and for slow spindles (Landolt and Borbély 2001); a higher spindle frequency in older subjects (Principe and Smith 1982; Crowley et al. 2002; Ktonas et al. 2007); higher fast spindle amplitudes and frequencies in females (Bódizs 2017); increase of spindle occurrence with learning demand (Gais et al. 2002; Eschenko et al. 2006; Schmidt et al. 2006; Schabus et al. 2008; Mölle et al. 2009); and a positive linear relationship between spindle occurrence and recall success for spindles measured between acquisition and recall (Gais et al. 2002; Clemens et al. 2005, 2006; Schmidt et al. 2006; Cox et al. 2012; Seeck-Hirschner et al. 2012; Lustenberger et al. 2016; Hahn et al. 2018; Kuula et al. 2019).

[37] The segments of interest can, however, be defined even more narrowly in humans, since in humans non-REM sleep can be subdivided in 4 stages (see also Figure 2 in the introduction).

Indirect evidence for a similar involvement of sexual hormones, in particular progesterone, emerged from the observation that in dogs fast spindle density and frequency were higher in intact females compared to intact males and neutered females (Iotchev et al. 2017, 2019), and that the variation of fast spindle density was higher in the intact female than intact male group (Supplementary). Fast spindle density and frequency vary with the menstrual cycle in humans (Driver et al. 1996; Baker et al. 2007; De Zambotti et al. 2015) and peak during the high progesterone luteal phase (Baker and Driver 2007), suggesting that the values for density and frequency can be sometimes higher than in males, as also suggested by the observations of Gaillard and Blois (1981), Ackermann et al. (2015) and Bódizs (2017). In humans, a higher occurrence of spindles in women has been reported in a handful of studies, or only for sub-populations like major depression patients (Gaillard and Blois 1981; Plante et al. 2013; Ackermann et al. 2015), but there are also conflicting findings (Ujma et al. 2014; Bódizs 2017).

Partial similarities include a more posteriorly pronounced decline in slow spindle density in the dog (Iotchev et al. 2019). In humans a posterior decline in spindle occurrence has been observed in pathological (Gorgoni et al. 2016), but not in healthy aging (Martin et al. 2012). Moreover, the decline in occurrence in pathological aging is more specific to the fast spindle subtype in humans. Subjects in Iotchev et al. (2019) were all reported as healthy by the owners, however, which has to be taken into account when comparing with human-specific findings. The observation of more abundant fast spindles in older dogs better resembles a similar shift from childhood to adolescence in humans (Hahn et al. 2018), but is better classified as a species-specific observation than a similarity[38]. It is also a curious observation that amplitudes of slow spindles, measured over Cz, appear to rise with age in females (Iotchev et al. 2019). This does not align with what we know about the mechanisms underlying spindle amplitude and how they are affected by aging (Guazzelli et al. 1986; Sitnikova 2010).

The total sum of similar predictive relationships obtained from the studies reported here (Iotchev et al. 2017, 2019) suggest an overall fulfillment of the predictive validity criteria. The fact that some observations are more unique to dogs are not surprising, as some species-specific adaptations are expected in every analogous or homologous trait. For example, rats also do not show some of the spindle characteristics observed in humans, such as learning-dependent coupling of spindles and slow oscillations or changes induced by the menstrual cycle on how spindles are expressed (Schwierin et al. 1998; Mölle et al. 2009). The number of similarities

[38] Given the life-span of each species the human observation is more likely a sign of development and the observation in dogs a sign of aging.

between human and dog spindles, spanning across associations with learning (Iotchev et al. 2017), aging (Iotchev et al. 2019), and sexual dimorphism (Iotchev et al. 2017, 2019) together form a strong argument for high predictive and external validity.

6.3.3 Construct validity

Construct validity is fulfilled when the model and modeled phenomenon share similar causal structures (van der Staay et al. 2009). A strong proof for construct validity in model animals used in comparative spindle research is provided by the cat (Steriade and Llinás 1988) and to some extend the rat (Kandel and Buzsáki 1997; Meeren et al. 2009; Peyrache et al. 2011), where mechanisms have been studied with invasive recordings and lesions. In a pet animal model, which is in the care and protection of an owner, such a procedure is not applied easily, as even pharmacological interventions would require negotiation with and consent from the owners.

However, I was able to bypass this hurdle by analyzing the effects of neutering, a common surgical intervention that dramatically alters the hormonal levels of pet dogs, and comparing them to the known effects of hormonal variations in humans (Driver et al. 1996; Baker and Driver 2007; Baker et al. 2007; De Zambotti et al. 2015), thus inferring potential mechanistic similarities between the two species. The interaction between sex and reproductive status on the expression of (fast) spindle density and frequency observed in Iotchev et al. (2019) suggests some shared mechanisms with human spindles, which are also under the influence of sexual hormones (Driver et al. 1996; Baker and Driver 2007), but so are other processes in the mammalian brain (for instance in rats, the female sexual hormone progesterone affects the activity of four different neurotransmitters (Chaudhury et al. 1992)). Moreover, while pharmacological responding does indirectly inform about mechanisms, it is usually discussed in the literature as an argument for predictive validity (Overall 2000; van der Staay 2006). The anterior decline in amplitude is also not exclusive to spindles (Landolt and Borbély 2001), although potentially it is a relevant marker of underlying causal dynamics (Guazzelli et al. 1986; Sitnikova 2010).

Any arguments that can be made about the construct validity of the dog model therefore depend in parts on our conclusions about the strength of the face- and predictive validities in suggesting that we really observe sleep spindles in the dog. In light of above arguments for face, external and predictive validity, similar differences between the sexes, topographic expression and animals of different age indirectly suggest the involvement of similar mechanisms in spindle generation, yet conclusive evaluation of the canine model's construct validity would require

invasive work which is not likely in the pet model and might more generally disappear as an option for dogs (Bailey and Pereira 2018). Neutering, however, as a common invasive procedure in companion animals presents an important exception to our limitations in working with pet models.

6.3.4 Conclusions on the dog as a model animal

I have found converging evidence that dogs express sleep spindles and that the method I developed to detect these electrophysiological events is adequate and effective. More specifically, we were able to obtain direct and indirect arguments for both face- and predictive validity, from which by extension we also obtained indirect arguments for the canine model's construct validity (of which the strongest supportive argument is the effects of neutering). We conclude that the convergent evidence is strong enough to support the more unexpected findings, like age-related increases in frontal fast spindle density and (for females) central slow spindle amplitude.

The dog offers several unique advantages as a model animal in comparative sleep spindle research, despite invasive methods being a less welcome approach in this species (Bailey and Pereira 2018). These are a shared environment with humans and a relatively complex social cognitive repertoire (Topál et al. 2009), combined with a shorter life-span easing longitudinal investigations. Considering that effects of development (Petersen et al. 1964) and aging (Iotchev et al. 2019) were observed to affect sleep spindles in dogs, but not as much in rats (van Luijtelaar and Bikbaev 2007; Sitnikova et al. 2014a), we conclude that dogs are more likely to receive further attention as model animals for the aging, sleeping brain. Canine spindles also appear more humanlike than rat spindles with regard to how hormones might shape spindle expression (Schwierin et al. 1998). Last, but not least, dogs are themselves the target of veterinary care and thus also act as models for their own conditions. The more frequently studied canine epilepsy (Pellegrino and Sica 2004; Jeserevics et al. 2007; Aiello et al. 2012; Pákozdy et al. 2012) faces important limitations, for epilepsy in general cannot always be traced to a specific mechanism or location in the brain (Blumenfeld and Taylor 2003), whereas converging evidence from cats, rats and humans suggests a universal thalamic (specifically RTN) origin of spindles in species that express them (Steriade and Llinás 1988; Schabus et al. 2007; Meeren et al. 2009).

6.4 Conclusions for the evolution of the (sleeping) mind-brain (in dogs and beyond)

Below we will turn to discuss how the here presented findings promote our understanding of dog cognition and evolution both within and beyond the narrow context of sleep physiology,

as well as how these findings in the dog promote our understanding of sleep and sleep spindles more generally.

6.4.1 The canine spindle in an evolutionary context

Sleep spindles have not been observed in non-mammalian clades (Rattenborg and Martinez-Gonzalez 2011; Rattenborg et al. 2011; Shein-Idelson et al. 2016; Van Der Meij et al. 2019) Even among mammals, for most species we have only observational evidence which can at best satisfy the criterion of face validity (Kryger et al. 2011). Aside from humans, we only had substantial evidence for the existence of real sleep spindles in the cat (Steriade and Llinás 1988) and rat (Terrier and Gottesmann 1978; Kandel and Buzsáki 1997; Siapas and Wilson 1998; Eschenko et al. 2006; Meeren et al. 2009; Mölle et al. 2009; Sitnikova 2010; Peyrache et al. 2011; Sitnikova et al. 2014b). The convergent evidence generated in this thesis for the existence of real sleep spindles in the dog brings us closer to the conclusion that spindles are a universal feature of mammalian sleep. It would be an interesting endeavor to investigate the potential existence of sleep spindles on more ancestral members of the mammalian clade, like marsupials or monotremes.

Evidence for changes in sleep spindle expression in dogs associated with development and aging (Petersen et al. 1964; Iotchev et al. 2019) compared to humans, suggests either an evolutionary change in how brains develop and age, or that mechanisms of spindle generation and/or shaping do not age relative to life-span. An increase in fast spindle occurrence is observed for humans, from childhood to adolescence (Hahn et al. 2018). If such a trajectory is not dependent on life-span, however, than only for fast spindles. In dogs, older animals show the same decline in slow spindle amplitude and density as humans and it is also localized (Landolt and Borbély 2001; Martin et al. 2012), albeit, as we saw above, not always in the exact same way.

That rat spindles seem unaffected by the estrous cycle (Schwierin et al. 1998), but in dogs the role of sexual hormones is strongly suggested by the effects of neutering (Iotchev et al. 2019). There is physiological evidence in support of phylogenetic reconstructions which place carnivores closer to humans (Cannarozzi et al. 2007) as opposed to those who claim that rodents and primates are more closely related (Murphy et al. 2001). As a note of caution to this interpretation, however, convergent evolution and specialized breeding sometimes produce humanlike physiology in dogs, for instance in the case of binocular and high acuity vision in brachycephalic dogs (McGreevy et al. 2004).

Dogs presented with verbal learning material showed an increase in spindle density and a linear association between spindle density and recall success specifically expressed for the slow subtype (Iotchev et al. 2017). Notably, spindle-learning associations in the human literature are also more likely associated with slow spindles or spindles detected frontally (where slow spindles predominate) when the subjects are engaged in verbal learning (Clemens et al. 2005; Schmidt et al. 2006; Schabus et al. 2008; Kuula et al. 2019), while motor learning is more reliably associated with fast and posterior spindles (Tamaki et al. 2009; Barakat et al. 2011; Astill et al. 2014; Lustenberger et al. 2016; Yordanova et al. 2017). The findings in Iotchev et al. (2017) align thus with other neuroimaging (Andics et al. 2016) and behavioral studies (Kaminski et al. 2004; Pilley and Reid 2011; Ramos and Ades 2012), suggesting that dogs process words similar to humans. A more cautious interpretation is that slow spindles support processes that are more relevant to verbal than motor learning, but that neither spindle type nor associated learning-mechanism is specific to the verbal, respectively motor domain in a strict sense. For instance, a more pronounced involvement of slow spindles in memory consolidation could reflect engagement with more abstract or difficult to memorize material (Schmidt et al. 2006; Schabus et al. 2008).

6.4.2 General conclusions on sleep spindles, their function and evolution

The here presented findings also enable us to draw some conclusions about sleep spindles in general, for which I will first turn to the last finding discussed under point 6.4.1. Although the paradigm in Iotchev et al. (2017) (earlier used in Kis et al. (2017b)) does not distinguish true verbal learning from alternative strategies in forming sound-action associations, the findings of a correlation between spindles and learning, specific to the slow type in this study, adds to the growing body of evidence that slow spindles support learning specifically when verbal material is used (Clemens et al. 2005; Schmidt et al. 2006; Schabus et al. 2008; Kuula et al. 2019). This association, moreover, seems to thus hold independent of the studied species[39], although to exclude simpler explanations future work should test what happens when action commands are paired with pure tones or other simpler sounds, instead of spoken words. Our findings also lend support for the utility of spindle occurrence to predict learning performance in general, a notion that has been critically challenged by the large-sample study of Ackermann et al. (2015). Our findings instead align with the more numerous positive results, and with mechanistic work supporting the importance of sleep spindles in memory consolidation

[39] Although it is plausible to demand that the studied animal should be capable of something similar to human verbal learning.

(Rosanova and Ulrich 2005; Latchoumane et al. 2017). In the future, however, more studies with large samples will be required to further clarify the controversy. Currently the gross of positive findings is based on sample-sizes equal or less than N = 20.

Our findings in the dog more generally support a distinction between fast and slow spindles. It seems that the localized distribution of both types is universal across mammals if we compare the observations from humans, rats and dogs (Gibbs and Gibbs 1961; Terrier and Gottesmann 1978; Iotchev et al. 2019), and that it follows a pattern of more abundant fast spindle expression over central and posterior parts of the cortex. In light of this, the observation that age and sexual dimorphism differentially affect slow and fast spindle features in dogs (Iotchev et al. 2019) support the notion that the two types are generated by different mechanisms (Ayoub et al. 2013).

Although some authors have proposed that only one of the subtypes is thalamic (Timofeev and Chauvette 2013), it is important to consider that processes both in the thalamus (Steriade and Llinás 1988; Schabus et al. 2007; Meeren et al. 2009) and in the cortex (Kandel and Buzsáki 1997; Sitnikova 2010; Peyrache et al. 2011) can contribute to the features of spindles as they appear in the surface EEG signal. Hence, it is also possible that slow and fast spindles only differ in cortical mechanisms. In support of the latter interpretation, early experimental work in cats found that the entire sigma range (in cats defined as 7-14 Hz) depends on the same thalamic mechanisms (RTN burst-mode firing, see Steriade et al. (1985, 1987)), while in humans, both slow and fast spindles are associated with thalamic activation (Schabus et al. 2007). Our work also supports the latter interpretation in that we were able to replicate in the dog many findings associated to fast and slow spindles in humans by initially applying the normal-modeling search procedure for the entire sigma range (9-16 Hz as defined by Bódizs et al. (2009)) and then dividing into subcategories only after the search was completed. This suggest that detections of both subtypes are part of the same normally distributed[40] (with regard to amplitude and frequency) population of transients.

Finally, our findings imply that the sigma frequency range is more similar between species than was once assumed. Different frequency ranges had been proposed for the sleep spindles of different mammals (Zepelin and Rechtschaffen 1974; Kryger et al. 2011), but most of these estimates were based on visual inspection. In contrast, we report that only detections obtained

[40] Although as Figure 13 suggests, the underlying normal distribution of spindle frequency is an abstraction (and certainly not visible when pooling across individuals).

with a human-like definition of sigma (9-16 Hz, Bódizs et al. (2009)) predict recall success in dogs (Iotchev et al. 2017), while detections in this frequency band also display other similarities with human and rat spindles (Iotchev et al. 2019). Given that prior to our work dogs were also assumed to have a dissimilar spindling frequency from humans (Kryger et al. 2011; Pákozdy et al. 2012), it is possible that previous conclusions that were solely based on visual inspection will need to be revised.

6.5 General conclusion and future directions

The present work set out to obtain answers to several interrelated questions at once. Do dogs display sleep spindle activity that resembles that of humans, rats and cats? Can we reliably quantify canine spindles and by doing so what do we learn about both the dog and the sleep spindle more generally?

The path we chose to address these questions was to collect a cross section of data that would allow for a conclusion based on a potential convergence of evidence. What was known about human, cat and rat sleep spindles suggested sleep spindles across species display similar functional correlates, such as an involvement in learning, which was demonstrated for both humans and rats (Mölle et al. 2009). No studies on this or other relationships had been described in dogs when I started my investigations. Furthermore, previous attempts at a systematic detection and quantification of canine spindles were not successful (Kis and Bódizs, personal communication).

My detection method and the analogies we observed between human and canine spindles mutually support each other. But for this to work without committing the fallacy of circular reasoning, it was important to keep the parameters of the detection algorithm close to criteria validated in humans, to compare its reliability and its ability to reproduce relationships known from the human literature across independent data sets[41], and to define a narrow set of starting hypotheses about the defining features of canine spindles on which to base the search.

The detection algorithm I developed for sleep spindle detection in dogs is reliable and validated by analogies in three domains associated with spindle activity (learning, aging, and sexual dimorphism) between human and dog spindles. These convergent arguments emerged across and within the two studies reported here and were interpreted using a generalized version of the three validities used in disease model validation (van der Staay et al. 2009). That sleep

[41] There is a minor overlap of subjects between the two studies reported here, but no overlap in polysomnographic recordings analyzed.

spindles were defined by the same detection criteria in both studies is also very important given the controversy of how comparable the currently circulating detection methods are in general (Warby et al. 2014). That the findings of both studies resonate with numerous observations in humans and rodents supports the construct validity of my instrument[42]. I also described a number of spindle-related discoveries unique to dogs which stimulate exciting questions for future research. One of them is the question of whether a higher number of fast spindles in older dogs resembles a similar development in young humans (Hahn et al. 2018) (life-span independent developmental trajectory) or if lower cortical inhibition, associated with aging (McQuail et al. 2015), results in more spindles with higher frequencies (see Steriade and Llinás (1988)).

An important future step is the further refinement of detection algorithms for canine sleep spindles. Although our method has been validated, even more accurate and automated measurements might be needed if sleep spindle detection is to become part of the veterinarian diagnostic toolkit.

List of publications

Publications related to the thesis

1. Iotchev, I. B., Kis, A., Bódizs, R., Van Luijtelaar, G., & Kubinyi, E. (2017). EEG transients in the sigma range during non-REM sleep predict learning in dogs. *Scientific reports*, *7*(1), 12936.
2. Iotchev, I. B., Kis, A., Turcsán, B., de Lara, D. R. T. F., Reicher, V., & Kubinyi, E. (2019). Age-related differences and sexual dimorphism in canine sleep spindles. *Scientific reports*, *9*(1), 10092.

Other publications of Ivaylo B. Iotchev

1. Iotchev, I. B., & van Schie, H. T. (2017). When a model becomes the real thing: A neuro-cognitive account of 'demonic' possession. *Medical hypotheses*, *106*, 35.
2. Bognár, Z., Iotchev, I. B., & Kubinyi, E. (2018). Sex, skull length, breed, and age predict how dogs look at faces of humans and conspecifics. *Animal cognition*, *21*(4), 447-456.

[42] Not to be confused with construct validity as discussed in the animal model literature, here I refer to the ability of an instrument to measure what it claims to measure.

3. Iotchev, I. B., & Costa, K. M. (2019). Animal cognition: Quantity has a quality of its own. *Animal Sentience*, *3*(23), 44.
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5. Wallis, L. J., Iotchev, I. B., Kubinyi, E. (2019). Demographic variables and personality traits as predictors of perceived dominance status in dogs. *PLoS One* (in press)
6. Iotchev, I. B., Szabó, D., Kis, A., & Kubinyi, E. (in review). Spindle frequency is a stable biomarker of reversal-learning in aged family dogs.

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Supplementary

Supplementary to Chapter 3

Absolute performance on the learning task

Dog	correct trials – control	correct trials – test	correct trials – retest
Álmos	16	11	12
Csinszka	18	9	12
Dorisz	16	12	12
Füles	14	12	11
Glenn	15	10	15
Grog	14	12	13
Kendra	15	9	13
Kenny	18	17	18
Maya	16	11	15
Naty	17	11	13
Onix	12	12	12
Rumli	16	13	13
Smafu	16	10	12
Tódor	17	16	17
Wicca	15	12	15

Table S1. Number of correct trials (from a total of 18) for each dog and session. Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

Additional results, transients in the 12-14 Hz and 5-12 Hz range

Analysis effects of age, sex and learning on spindle density in the learning condition. Below the results for the 12-14 Hz and 5-12 Hz transients:

1. 12-14 Hz transients: The density of detections (spindles/minute) in the learning condition was not affected by learning gain (GLMM, $F_{1,10} = 0.322$, $P = 0.583$) or age (GLMM, $F_{1,10} = 3.028$, $P = 0.112$), but was predicted by sex (GLMM, $F_{1,10} = 26.698$, $P < 0.001$). Females displayed more spindles per minute than males (0.99 ± 0.1 versus 0.36 ± 0.06, means ± SE, $t_{10} = 4.834$, $P = 0.001$).

2. 5-12 Hz transients: There was no effect of learning gain (GLMM, $F_{1,11} = 0.004$, $P = 0.95$) or age (GLMM, $F_{1,11} = 0.656$, $P = 0.435$), but again the effect of sex was significant (GLMM, $F_{1,11} = 8.656$, $P = 0.013$) with females exhibiting more spindles/minute than males (12.67 ± 0.5 versus 7.61 ± 1.4, means ± SE, $t_{11} = 3.376$, $P = 0.006$).

Local spectral peaks in the dogs' alleged spindle-frequency

Although an absolute cut-off frequency has been used in previous work on humans, to delineate slow from fast spindles (13 Hz in Schabus et al. (2006)), we further inquired if such a cut-off correctly describes the distribution of frequency power in the dogs. To this end we looked for local peaks in the power of each dog's estimated spindle-frequency range, based on the 9-16 Hz search-criterion. The estimated spindle-frequency range (see methods) was the average frequency of the detections +/- 2 standard deviations. Results are shown in Table S2. In addition, we present, across animals, a distribution histogram of detections per frequency for each the control and learning condition (Figure S1).

Dog	spectral peaks sigma (control condtion):	spectral peaks sigma (learning condition):
Álmos	8.7 Hz	8 Hz
Csinszka	14.1 Hz	9 Hz
Dorisz	8.7 Hz	9.6 Hz, 13.1 Hz
Füles	12.4 Hz	9.8 Hz
Glenn	8.6 Hz	9.3 Hz
Grog	9.9 Hz	8.9 Hz
Kendra	9.1 Hz	8.7 Hz
Kenny	8.8 Hz	8.6 Hz
Maya	8.8 Hz	8.8 Hz
Naty	9 Hz	9.3 Hz
Onix	12.7 Hz	8.9 Hz
Rumli	9.9 Hz	9.9 Hz
Smafu	8.9 Hz	9.1 Hz, 14.7 Hz
Tódor	8.9 Hz	8.5 Hz
Wicca	7.9 Hz	8.4 Hz

Table S2. Local peaks in the power of the estimated sigma-range of each dog, listed for each dog and session.

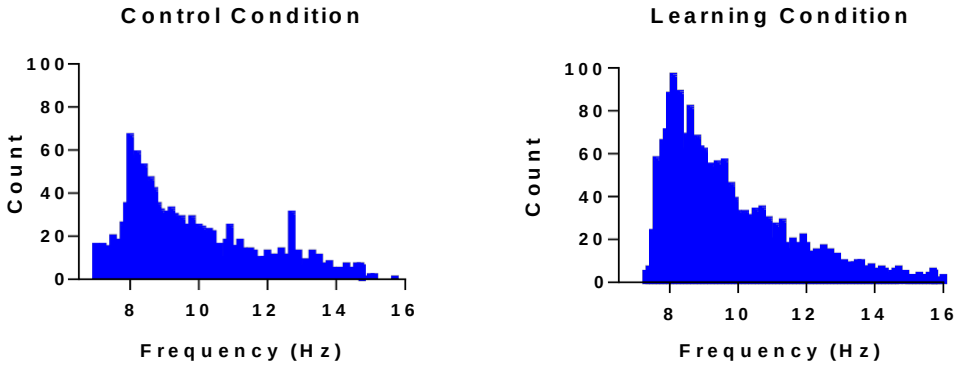

Figure S1. Count of detections, per frequency and across animals, marked as spindle-containing time windows in the 9-16 Hz search for each the control and learning condition. Adopted from Iotchev et al. (2017).

Examples of spindle events, slow (<= 13 Hz) and fast (>= 13 Hz)

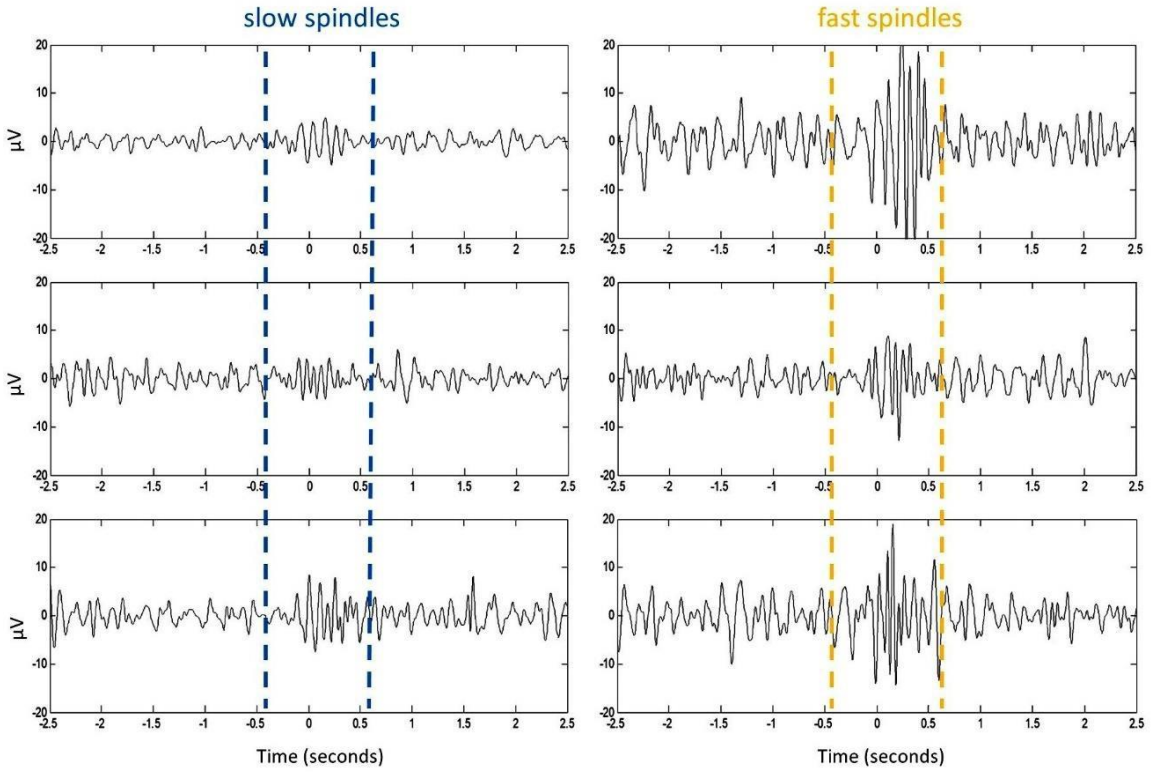

Figure S2. Examples of various detections in the 9-16 Hz range, classified either as 'slow-' or 'fast spindles'. Shown are 2.5 seconds of the filtered signal before and after time point of detection, for amplitude values between 20 and -20 µV. Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

Fast spindles, mean amplitude as a predictor of age

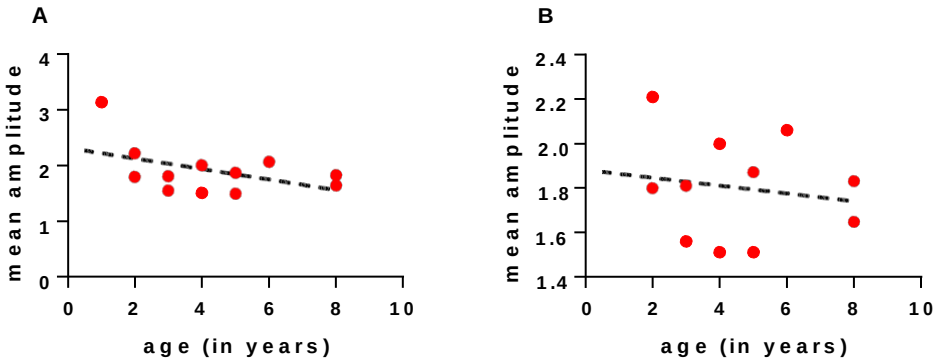

Figure S3. There was an association between age (in years) and the mean amplitude of fast spindles (A), however this effect seemed dependent on a single observation and disappeared when the data point was removed (B). The scaling in (A) and (B) is adjusted to the maximum values. Adopted from Iotchev et al. (2017). Sharing allowed under the Creative Commons license http://creativecommons.org/licenses/by/4.0/.

Control Analyses

To ensure that any effects observed were due to activity in the targeted frequencies and not artefacts caused by their harmonics, we repeated the above comparisons with the first lower and the first higher harmonic of the 9-16 Hz range. We focused on this range, because effects of learning and age were only found for 9-16 Hz transients, whereas sex differences were found in every range. In other words, there were no effects specific to the 5-12 Hz or 12-14 Hz detections. The higher harmonic, 18-32 Hz, was tested with 0.25 seconds long time windows and events were considered separate if they were more than 0.25 seconds apart. The lower harmonic, 4.5-8 Hz, was tested with one-second-long time windows and detections were considered separate events if more than a second apart.

1. In the learning condition, density of detections in the lower harmonics was not predicted by learning gain (GLMM, $F_{1,11} = 0.232$, $P = 0.64$) or age (GLMM, $F_{1,11} = 0.48$, $P = 0.503$), but there was an effect of sex (GLMM, $F_{1,11} = 9.744$, $P = 0.01$). Females displayed a higher density than males (5.7 ± 0.2 versus 3.2 ± 0.6, means $\pm$ SE, $t_{11} = 3.681$, $P = 0.004$).

2. Density of detections in the higher harmonics, during the learning condition, did not correlate with learning gain (GLMM, $F_{1,6} = 1.376$, $P = 0.285$) or sex (GLMM, $F_{1,6} =$

1.964, P = 0.211), but increased with age (GLMM, $F_{1,6}$ = 7.032, P = 0.038). The effect of age was not significant in post-hoc testing (GLMM, $F_{1,8}$ = 3.306, P = 0.107).

We also repeated the between condition comparisons with each harmonic. There was no difference in density between the learning and control condition for detections in the lower harmonics (t_{14} = 0.674, P = 0.511) or higher harmonics (t_{14} = 0.033, P = 0.974).

To control for alpha activity, we also ran our analyses with the target range 7.5-12.5 Hz, previously used in dogs to measure alpha (Wauquier et al. 1988). We found no effect of learning gain (GLMM, $F_{1,11}$ = 1.22, P = 0.293), age (GLMM, $F_{1,11}$ = 0.02, P = 0.891) or sex (GLMM, $F_{1,11}$ = 0.494, P = 0.497) for the density of detections in the learning condition. There was also no difference between the control and learning condition (t_{14} = 0.958, P = 0.354).

One final control was to exclude animals whose mean amplitude was more than 2 standard deviations above baseline. Although our algorithm restricts the final count to those events with an amplitude within 2 standard deviations of all initial detections, we reasoned that outliers would more easily bypass this control if an animal has overall too few detections in a given session. In the learning condition only one animal's mean amplitude exceeded 2 standard deviations. Excluding this dog, the spindle density was still significantly rising with age (GLMM, $F_{1,10}$ = 11.899, P = 0.006), learning gain (GLMM, $F_{1,10}$ = 12.640, P = 0.005), and was different for the sexes (GLMM, $F_{1,10}$ = 11.120, P = 0.008); Females displayed a higher density than males (4.8 ± 0.2 versus 3 ± 0.3, means ± SE, t = 5.03, P = 0.001). Learning gain remained significant in post-hoc testing (GLMM, $F_{1,12}$ = 9.386, P = 0.01), but age had no effect as a sole predictor (GLMM, $F_{1,12}$ = 0.38, P = 0.549). In the control condition three more dogs were excluded for displaying a mean amplitude exceeding 2 standard deviations. There was still a trend for a higher density in the learning condition (4.3 ± 0.4 versus 3.6 ± 0.4, means ± SE, t_{10} = 1.838, P = 0.096), but this difference was now only significant for dogs with less than 10 days waiting time between the tests (4.4 ± 0.6 versus 3.1 ± 0.6, means ± SE, t_6 = 3.67, P = 0.01).

Supplementary to Chapter 4

Descriptives

Time spend awake (M ± SD; range): 73.5 ± 40.2; 11.3 – 181.7 (minutes)

Time in drowsiness (M ± SD; range): 42.5 ± 24.2; 0 – 122.7 (minutes)

Time in non-REM (M ± SD; range): 39.6 ± 26.8; 0 – 121.3 (minutes)

Time in REM (M ± SD; range): 21.7 ± 19.8; 0 – 80.7 (minutes)

Control analyses

For the full-sigma range (9-16 Hz) there was no difference in density (t_{145} = 1.064, P = 0.289), amplitude (t_{144} = 0.563, P = 0.574) or frequency (t_{144} = 0.041, P = 0.968) of the detections between dogs measured with different settings (see methods). For fast spindles (≥ 13 Hz) there was no difference in density (t_{145} = 0.704, P = 0.483), amplitude (t_{125} = 0.925, P = 0.357) or frequency (t_{125} = 0.182, P = 0.856) between recordings obtained with different settings. There was also no difference between dogs recorded with different settings regarding the density (t_{145} = 0.931, P = 0.354), amplitude (t_{143} = 0.705, P = 0.482) or frequency (t_{143} = 0.116, P = 0.908) of slow (≤ 13 Hz) spindles.

We tested for demographic differences (age, sex, reproductive status) in breed groups to exclude confounds between breed and demographics as potentially competing predictors of spindling features. Breed groups with at least 8 individuals were Border Collies (N = 14), Golden Retrievers (N = 9), and Hungarian Vizslas (N = 8). These breed groups did not differ significantly with regards to age (ANOVA, F_2 = 1.635, P = 0.213), sex composition (z-test, P > 0.05) or reproductive status (z-test, P > 0.05).

We next tested if the largest breed-matched samples differed in spindling measures – density (spindles/minute), frequency and amplitude (for detections in the 9-16 Hz range). We found no difference in density (ANOVA, F_2 = 1.776, P = 0.188) and amplitude (ANOVA, F_2 = 0.057, P = 0.945), but there was a significant difference in frequency (ANOVA, F_2 = 4.742, P = 0.017). Hungarian Vizslas displayed higher spindle frequencies than Border Collies (mean difference = 0.7, P = 0.01) and Golden Retrievers (mean difference = 0.8, P = 0.012).

Final GLM models

Final model for spindle density (full sigma-range, 9-16 Hz) on Fz

predictors	Wald Chi-Square	P-values
sex	0.49	0.484
age	1.976	0.16
reproductive satus	1.298	0.255
sex x reproductive status	3.076	0.079

Final model for spindle density (full sigma-range, 9-16 Hz) on Cz

predictors	Wald Chi-Square	P-values
age	**4.940**	**0.026**

Final model for fast (≥ 13 Hz) spindle density on Fz

predictors	Wald Chi-Square	P-values
sex	**6.614**	**0.01**
age	**8.107**	**0.004**
reproductive status	0.002	0.964
sex x reproductive status	**8.351**	**0.004**

Final model for fast (≥ 13 Hz) spindle density on Cz

predictors	Wald Chi-Square	P-values
sex	**5.588**	**0.018**
age	1.425	0.233
reproductive status	0.533	0.465
sex x age	2.515	0.113
sex x reproductive status	2.294	0.130

Final model for slow (≤ 13 Hz) spindle density on Fz

predictors	Wald Chi-Square	P-values
reproductive status	**3.855**	**0.05**

Final model for slow (≤ 13 Hz) spindle density on Cz

predictors	Wald Chi-Square	P-values
age	**7.4**	**0.007**

Final model for spindle amplitude (full sigma-range, 9-16 Hz) on Fz

predictors	Wald Chi-Square	P-values
sex	0.389	0.533
age	**4.117**	**0.042**
sex x age	2.144	0.143

Final model for spindle amplitude (full sigma-range, 9-16 Hz) on Cz

predictors	Wald Chi-Square	P-values
sex	2.652	0.103
age	**4.351**	**0.037**
sex x age	**4.998**	**0.025**

Final model for fast (≥ 13 Hz) spindle amplitude on Fz

predictors	Wald Chi-Square	P-values
sex	**5.724**	**0.017**

Final model for fast (≥ 13 Hz) spindle amplitude on Cz

predictors	Wald Chi-Square	P-values
sex	0.191	0.662
reproductive status	0.618	0.432
sex x reproductive status	2.877	0.09

Final model for slow (≤ 13 Hz) spindle amplitude on Fz

predictors	Wald Chi-Square	P-values
sex	0.397	0.528
age	**4.169**	**0.041**
sex x age	1.594	0.207

Final model for slow (≤ 13 Hz) spindle amplitude on Cz

predictors	Wald Chi-Square	P-values
age	4.07	0.044
sex	3.6	0.058
sex x age	**5.685**	**0.017**

Final model for spindle frequency (full sigma-range, 9-16 Hz) on Fz

predictors	Wald Chi-Square	P-values
reproductive status	1.606	0.205

Final model for spindle frequency (full sigma-range, 9-16 Hz) on Cz

predictors	Wald Chi-Square	P-values
sex	**8.594**	**0.003**
age	1.705	0.192
reproductive status	0.009	0.923
sex x age	**4.252**	**0.039**
sex x reproductive status	**5.615**	**0.018**

Final model for fast (≥ 13 Hz) spindle frequency on Fz

predictors	Wald Chi-Square	P-values
sex	**5.394**	**0.02**
reproductive satus	1.085	0.298
sex x reproductive status	**4.014**	**0.045**

Final model for fast (≥ 13 Hz) spindle frequency on Cz

predictors	Wald Chi-Square	P-values
age	**5.666**	**0.017**
reproductive status	**5.343**	**0.021**

Final model for slow (≤ 13 Hz) spindle frequency on Fz

predictors	Wald Chi-Square	P-values
sex	1.682	0.195

Final model for slow (≤ 13 Hz) spindle frequency on Cz

predictors	Wald Chi-Square	P-values
sex	**9.852**	**0.002**
age	0.457	0.499
reproductive status	0.393	0.531
sex x age	**6.023**	**0.014**
sex x reproductive status	**4.406**	**0.036**

Differences in variance

The tests of equal variance available in GraphPad Prism were used to compare variance differences between male and female dogs, as well as neutered and intact animals in our post-hoc tests. The variance for fast spindle density on Fz was greater for neutered than intact male dogs ($F = 7.719$, $P < 0.001$). Among intact animals, females showed a greater variance for fast spindle density on Fz than males ($F = 13.37$, $P < 0.001$). There was no difference in fast spindle density variance between female and male dogs on Cz ($F = 1.493$, $P = 0.2123$).

Fast spindle amplitude variance was greater for females than males on Fz ($F = 27.35$, $P < 0.001$).

Spindle frequency variance on Cz was not different between intact males and females, but there was a trend for males to display a greater variance ($F = 6.158$, $P = 0.0956$). There was no difference between intact female and male dogs' variance of slow spindle frequency ($F = 2.587$, $P = 0.3735$). The variance of fast spindle frequency on Fz was also not different between intact females and intact males ($F = 2.394$, $P = 0.1604$), nor between intact and neutered females ($F = 1.489$, $P = 0.5103$) for detections on Cz from the full sigma-range. The variance of neutered females compared to intact females was, however, higher for fast spindle frequency on Cz ($F = 9.891$, $P = 0.0371$).

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I. The data of the doctoral dissertation:
Name of the author: Ivaylo Borislavov Iotchev
MTMT-identifier: 10063778
Title and subtitle of the doctoral dissertation: The dog as a model-animal in comparative sleep spindle research
DOI-identifier[72]:
Name of the doctoral school: Doctoral School of Biology
Name of the doctoral programme: Doctoral Program of Ethology
Name and scientific degree of the supervisor: Enikő Kubinyi PhD
Workplace of the supervisor: Eötvös Loránd University, Department of Ethology 1117 Budapest, Pázmány Péter sétány 1/c

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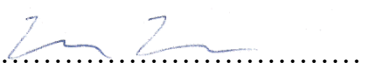

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